Morton City Council 17 May, 2021 A.D.

I have the fear of the LORD, Let it be contagious.
No sharia burqa slave mask mandates; No genetic modified serum injection "vaccine" mandates;
No covid green passports mandates

Michael D. Jackson
100 Caroline Street
Morton, Illinois

• Goodly Heritage, Posterity; born again Christian; natural born American Citizen; Registered Nurse

• **God's Word violated**: Created in the Image of God (Genesis 1:27 KJB); not good that man should be alone socially isolated/distanced (Genesis 2:18 KJB) Christ's light is not to be hid under a bushel/mask (Matthew 5:14 KJB); [my] "body is the temple of the Holy Ghost which is in [me] (I Corinthians 6:19 KJB)

• **God's Laws Of Nature violated**: Sunlight is Vitamin D3; Lepers are quarantined and only for 14 days not the healthy (Leviticus 13,14 KJB); Vitamin C and Zinc are essential, just as sunlight, water and oxygen and you can't get it if you are in a lock downed prison house, masked up under dictatorial control, and being told you will have to get a genetic modified serum injection to buy or sell and participate in life freely and peacefully

• **Man's law under God violated**: United States Constitution, (1st, 4th, 10th, and 14th) ADA, HIPPA, OHSA, and Statutory Law USC 18 § 242 "depravation of rights under color of law"

• **Nuremberg Code violated**. The first and foremost principle is unequivocal: "*The voluntary consent of the human subject is absolutely essential*".

• J.B. "Jackboot" Pritzker's executive order ruled out of order "Void Ab Initio"

• **Face mask studies**: NEJM (Harvard), Stanford; **1918 Spanish Flu:** NIAID; **Vaccine studies:** Univ. Texas, Tulane & NYU

• FOIA: Morton Police Department Event Report (Cops with bandit masks going to arrest me for trespassing not wearing a face diaper into CU I'm a member of since my childhood)

• Thomas Jefferson: "If people let government decide what foods they eat and medicines they take, their bodies will soon be in as sorry a state as are the souls of those who live under tyranny."

• II Chronicles 7:14 KJB; Matthew 10:28 KJB; Romans 13:3,4 KJB

https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_id=25743&p_table=INTERPRETATIONS
https://www.osha.gov/sites/default/files/publications/osha3021.pdf
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https://fee.org/articles/youth-depression-suicide-increasing-during-pandemic-response/ https://fee.org/articles/harvard-study-an-epidemic-of-loneliness-is-spreading-across-america/

FACE MASKS
Pose Serious Risks To The Healthy

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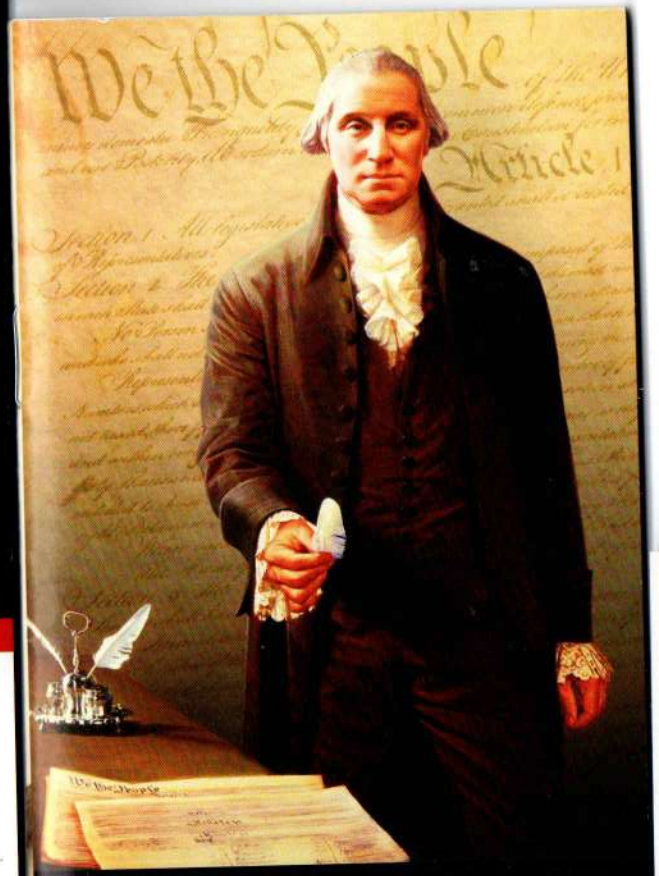
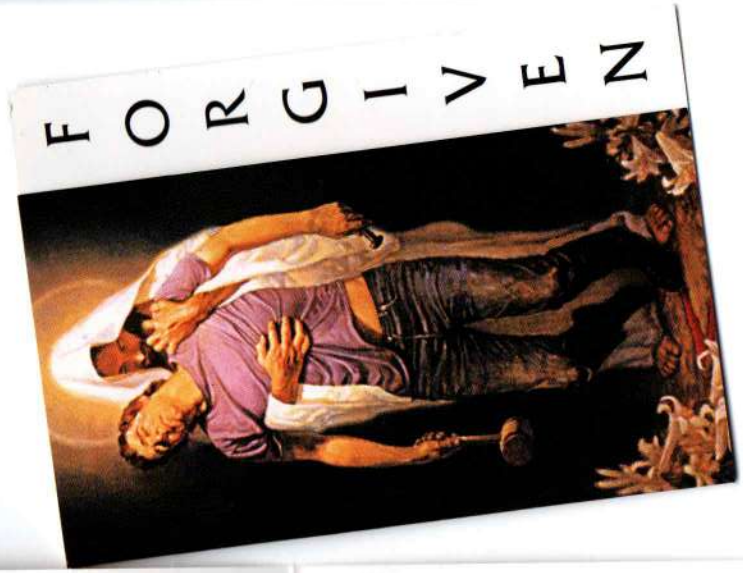
The Bible Way To Heaven

We Are All Under Sin "...There is none righteous, no, not one." (Rom 3:10) "For all have sinned, and come short of the glory of God;" (Rom 3:23)
There is a penalty for sin – "For the wages of sin is death;" – (Rom 6:23a)
Believe Christ died for you – "But God Commendeth his love toward us, in that, while we were yet sinners, Christ died for us." (Rom 5:8)
Jesus Wants You To Get Saved Today – "That if thou shalt confess with thy mouth the Lord Jesus, and shalt believe in thine heart that God hath raised him from the dead, thou shalt be saved." (Rom 10:9)
ACCEPT CHRIST AND HIS PAYMENT TODAY. PRAY FROM YOUR HEART...
Dear Lord, I confess that I am a sinner and I need to be saved. I believe that Jesus died on the cross to pay my sin debt. Forgive my sins, come into my life and save my soul. Amen.



THE CONSTITUTION OF THE UNITED STATES

...its only keepers, the people.
—George Washington



USE THIS PICTURE AS A WITNESSING TOOL BY MEMORIZING THESE 12 KEY VISUAL SYMBOLS

1. The painting's dark background signifies sin. It is the world of darkness within us that the light of Jesus Christ seeks to invade.

2. The mallet and the spike in the man's hands are reminders that each of us is responsible for the death of Jesus Christ on the cross.

3. The contemporary man in the painting is in despair and ready to fall to the ground. His expression carries the pain of sin, and his helplessness reflects total dependence on Jesus Christ for his salvation.

4. The figure upholding the repentant man is a picture of God's grace. He is the ever-present Jesus, ready to receive and redeem all who have been broken by the power of sin.

5. The setting of Forgiven is Mt. Calvary, the place where Jesus was crucified. It is here that He died to redeem us to God. It is here that each sinner must come to be forgiven.

6. Jesus' hands are slightly oversized, showing strength. They remind us that we cannot rely on our own strength; we are totally dependent upon the Lord.

7. The halo of light around Jesus' head signifies His holiness and divinity.

8. The wounded hand of Jesus over the man's heart upholds him and reveals God's desire to change all hearts by the power of His love.

9. The white robe that Jesus is wearing speaks to us of his righteousness. It is the garment with which we must all be clothed.

10. The blood that flows from Jesus' feet signifies the washing away of our sin and our justification before God.

11. The lilies in Forgiven remind us of the Lord's beauty and our worship and adoration of Him, the Lily of the Valley and the Rose of Sharon.

12. The lilies also represent our personal growth in the Lord. Because He is alive in us, His life will produce fruit for His glory.

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FORGIVEN

https://www.ada.gov/ada_intro.htm?ncid=edlinkushpmg00000313

The Americans with Disabilities Act (ADA) was signed into law on July 26, 1990, by President George H.W. Bush. The ADA is one of America's most comprehensive pieces of civil rights legislation that prohibits discrimination and guarantees that people with disabilities have the same opportunities as everyone else to participate in the mainstream of American life -- to enjoy employment opportunities, to purchase goods and services, and to participate in State and local government programs and services. Modeled after the Civil Rights Act of 1964, which prohibits discrimination on the basis of race, color, religion, sex, or national origin – and Section 504 of the Rehabilitation Act of 1973 -- the ADA is an "equal opportunity" law for people with disabilities.

To be protected by the ADA, one must have a disability, which is defined by the ADA as a physical or mental impairment that substantially limits one or more major life activities, a person who has a history or record of such an impairment, or a person who is perceived by others as having such an impairment. The ADA does not specifically name all of the impairments that are covered.

https://www.ada.gov/regs2010/titleII_2010/titleII_2010_regulations.htm#a35108

§ 35.108 Definition of disability

(1) Physical or mental impairment means:

(i) Any physiological disorder or condition, cosmetic disfigurement, or anatomical loss affecting one or more body systems, such as: neurological, musculoskeletal, special sense organs, respiratory (including speech organs), cardiovascular, reproductive, digestive, genitourinary, immune, circulatory, hemic, lymphatic, skin, and endocrine; or

§ 35.130 General prohibitions against discrimination

(a) No qualified individual with a disability shall, on the basis of disability, be excluded from participation in or be denied the benefits of the services, programs, or activities of a public entity, or be subjected to discrimination by any public entity.

(b)

(1) A public entity, in providing any aid, benefit, or service, may not, directly or through contractual, licensing, or other arrangements, on the basis of disability—

(i) Deny a qualified individual with a disability the opportunity to participate in or benefit from the aid, benefit, or service;

(ii) Afford a qualified individual with a disability an opportunity to participate in or benefit from the aid, benefit, or service that is not equal to that afforded others;

(iii) Provide a qualified individual with a disability with an aid, benefit, or service that is

Occupational Safety and Health Administration's (OSHA's) Directorate of Enforcement Programs regarding the Respiratory Protection Standard, 29 CFR 1910.134.

Paragraph (d)(2)(iii) of the Respiratory Protection Standard considers any atmosphere with an oxygen level below 19.5 percent to be oxygen-deficient and immediately dangerous to life or health. To ensure that employees have a reliable source of air with an oxygen content of at least 19.5 percent, paragraphs (d)(2)(i)(A) and (d)(2)(i)(B) of the Respiratory Protection Standard require employers working under oxygen-deficient conditions to provide their employees with a self-contained breathing apparatus or a combination full-facepiece pressure-demand supplied-air respirator with auxiliary self-contained air supply. In the preamble to the final Respiratory Protection Standard, OSHA discussed extensively its rationale for requiring that employees breathe air consisting of at least 19.5 percent oxygen. The following excerpt, taken from the preamble, explains the basis for this requirement:

Human beings must breathe oxygen . . . to survive, and begin to suffer adverse health effects when the oxygen level of their breathing air drops below [19.5 percent oxygen]. Below 19.5 percent oxygen . . . , air is considered oxygen-deficient. At concentrations of 16 to 19.5 percent, workers engaged in any form of exertion can rapidly become symptomatic as their tissues fail to obtain the oxygen necessary to function properly (Rom, W., Environmental and Occupational Medicine, 2nd ed.; Little, Brown; Boston, 1992). Increased breathing rates, accelerated heartbeat, and impaired thinking or coordination occur more quickly in an oxygen-deficient environment. Even a momentary loss of coordination may be devastating to a worker if it occurs while the worker is performing a potentially dangerous activity, such as climbing a ladder. Concentrations of 12 to 16 percent oxygen cause tachypnea (increased breathing rates), tachycardia (accelerated heartbeat), and impaired attention, thinking, and coordination (e.g., Ex. 25-4), even in people who are resting.

At oxygen levels of 10 to 14 percent, faulty judgment, intermittent respiration, and exhaustion can be expected even with minimal exertion (Exs. 25-4 and 150). Breathing air containing 6 to 10 percent oxygen results in nausea, vomiting, lethargic movements, and perhaps unconsciousness. Breathing air containing less than 6 percent oxygen produces convulsions, then apnea (cessation of breathing), followed by cardiac standstill. These symptoms occur immediately. Even if a worker survives the hypoxic insult, organs may show evidence of hypoxic damage, which may be irreversible (Exs. 25-4 and 150; also reported in Rom, W. [see reference in previous paragraph]).

(Federal Register, Vol. 63, p. 1159.) The rulemaking record for the Respiratory Protection Standard clearly justifies adopting the requirement that air breathed by employees must have an oxygen content of at least 19.5 percent. A lesser concentration of oxygen in employees' breathing air could endanger them physiologically and diminish their ability to cope with other hazards that may be present in the workplace. The rulemaking record also demonstrates that any workplace atmosphere controlled at or near your recommended minimal oxygen level of 100 mm of mercury at sea level (equivalent to about 13 percent oxygen at sea level) is not safe and healthful for all employees. Exposing employees to partial pressures of oxygen that approach 100 mm of mercury at sea level leaves them with no margin of safety from potentially debilitating effects, which could appear suddenly and without warning.

https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_id=25743&p_table=INTERPRETATIONS

Summary:

Section 242 of Title 18 makes it a crime for a person acting under color of any law to willfully deprive a person of a right or privilege protected by the Constitution or laws of the United States.

For the purpose of **Section 242**, acts under "color of law" include acts not only done by federal, state, or local officials within their lawful authority, but also acts done beyond the bounds of that official's lawful authority, if the acts are done while the official is purporting to or pretending to act in the performance of his/her official duties. Persons acting under color of law within the meaning of this statute include police officers, prisons guards and other law enforcement officials, as well as judges, care providers in public health facilities, and others who are acting as public officials. It is not necessary that the crime be motivated by animus toward the race, color, religion, sex, handicap, familial status or national origin of the victim.

The offense is punishable by a range of imprisonment up to a life term, or the death penalty, depending upon the circumstances of the crime, and the resulting injury, if any.

TITLE 18, U.S.C., SECTION 242

Whoever, under color of any law, statute, ordinance, regulation, or custom, willfully subjects any person in any State, Territory, Commonwealth, Possession, or District to the deprivation of any rights, privileges, or immunities secured or protected by the Constitution or laws of the United States, ... shall be fined under this title or imprisoned not more than one year, or both; and if bodily injury results from the acts committed in violation of this section or if such acts include the use, attempted use, or threatened use of a dangerous weapon, explosives, or fire, shall be fined under this title or imprisoned not more than ten years, or both; and if death results from the acts committed in violation of this section or if such acts include kidnapping or an attempt to kidnap, aggravated sexual abuse, or an attempt to commit aggravated sexual abuse, or an attempt to kill, shall be fined under this title, or imprisoned for any term of years or for life, or both, or may be sentenced to death.

https://www.justice.gov/crt/deprivation-rights-under-color-law

The 1947 Nuremberg Code is the most important legal document in the history of medical research ethics.

It established 10 foundational principles of ethical clinical research.

The first and foremost principle is unequivocal: "*The voluntary consent of the human subject is absolutely essential*".

It prohibits research to be conducted on human beings without the informed consent **of the individual**.

The significance of the Nuremberg Code is as follows:

The Nuremberg Code was formulated by prominent US government jurists in consultation with prominent US medical consultants.

- It had the multilateral agreement of the governments of the US, USSR, France and the UK;
- The Nuremberg Code extended human rights beyond the borders of individual countries;
- The right of Informed Consent is recognized in time of peace and in time of war.
- The Nuremberg Code provides legal justification to litigate violations of informed consent.
- Under the Nuremberg Code, responsibility for violations of informed consent rests upon individual doctors, government officials – and their aiders and abettors – each of who can be prosecuted for crimes against humanity.

https://medicalkidnap.com/2021/04/06/the-nuremberg-code-the-universal-right-of-informed-consent-to-medical-interventions-has-been-recognized-in-us-law-since-at-least-1914/

IN THE CIRCUIT COURT
FOR THE FOURTH JUDICIAL CIRCUIT
CLAY COUNTY, ILLINOIS

Darren Bailey, Plaintiff, vs. Governor Jay Robert Pritzker, in his official capacity. Defendant.	)	Case No. 2020-CH-06

ORDER

THIS CAUSE COMING TO BE HEARD for hearing on the Plaintiff's Motion for Summary Judgment on Counts I, II and III of Plaintiff's Amended Complaint, the Court having considered the pleadings, arguments of counsel, and having been otherwise apprised of matters with the record on Summary Judgment supplanted as ordered by the Court in the 7/2/20 record of proceedings.

IT IS HEREBY ORDERED:

1) Defendant's request to make an oral motion for summary judgment is considered and the request denied without prejudice to file a written motion for summary judgment.
2) Plaintiff's motion for summary judgment as to Count I is denied.
3) Plaintiff's motion for summary judgment as to Count II is granted as follows:
    a) The Court declares Defendant issued Proclamation #2, as defined in the amended complaint, and Proclamation #3, as defined in the amended

complaint, for the same occurrence or threat which gave rise to the issuance of Proclamation #1, as defined in the amended complaint, on March 09, 2020;

b) The Court declares the 30-days of emergency powers provided under Section 7 of the IEMAA provided to the Defendant to address the COVID-19, lapsed on April 08, 2020;

c) The Court declares any executive orders in effect after April 08, 2020 relating to COVID-19, and finding their authority under the emergency powers of Section 7 of the IEMAA are void ab initio.

4) Plaintiff's motion for summary judgment as to Count III is granted as follows:

a) The Court declares Defendant had no Illinois constitutional authority as Governor to restrict a citizen's movement or activities and/or forcibly close business premises in EO 32;

b) The Court declares that none of the cited provisions of the IEMAA in EO 32 delegated Defendant any authority to restrict a citizen's movement or activities and/or forcibly close business premises;

c) The Court declares the proper authority to restrict a citizen's movement or activities and/or forcibly close their business due to any public health risks has been expressly delegated to the Department of Health under the Illinois Department of Public Health Act and the County Code;

5) On Plaintiff's oral motion, Count IV of his complaint is dismissed with prejudice.

6) Plaintiff' oral request that his Amended Complaint be a representative action and apply to all citizens of the State of Illinois is granted

DATE: 7-2-20

ENTER: Michael D. McHaney
JUDGE

Prepared by:
Thomas G. DeVore
IL Bar No. 0630573
DeVore Law Offices, LLC
118 N. 2nd St.
Greenville, IL 62246
tom@silverlakelaw.com

FILED
OCT 19 2020
Clerk of the Circuit Court
38

IN THE CIRCUIT COURT FOR THE SEVENTH JUDICIAL CIRCUIT
SANGAMON COUNTY, ILLINOIS

IN RE: COVID-19 LITIGATION | Case No: 2020-MR-589

ORDER ON MOTION TO VACATE JULY 2, 2020 CLAY COUNTY ORDER

This matter comes on for hearing on the Governor's Motion to Vacate the July 2, 2020 Order for Lack of Jurisdiction in Darren Bailey's case in *Clay County, Case No: 2020-CH-6*. All parties were present by counsel, Mr. Bailey was present in person. Arguments of counsel were presented to the Court on October 14, 2020. The Court took the matter under advisement. After considering the pleadings, the record, and the arguments of counsel, the Court hereby FINDS:

It is undisputed that the Federal Court Order of Remand was entered on the Federal Court docket on June 29, 2020. The Governor argued that the Clay County Court did not have jurisdiction to hear the Motion for Summary Judgment on July 2, 2020 because the Order of Remand from the Federal Court was not received by the clerk of the circuit court until July 6, 2020. It is Mr. Bailey's position that the Clay County Court did have jurisdiction as the Federal Court lost jurisdiction as soon as the Order of Remand was entered.

If the reasoning in the *Hartlein v. Illinois Power Co.*[1] case is followed, the Clay County Court did not regain jurisdiction until July 6, 2020 when the actual physical copy of the Order of Remand was received by the circuit clerk. If the reasoning in *Eastern v. Canty*[2] is followed, the circuit court in Clay County did not have to wait for a technical order of remand to be received before proceeding and exercising jurisdiction to dispose of the case. Another Illinois Supreme Court case, *Van Dyke v. Illinois Commercial Men's Ass'n*[3] held that upon remandment of the

[1] *Hartlein v. Illinois Power Co.*, 155 Ill. 2d 142 (1992)
[2] *Eastern v. Canty*, 75 Ill. 2d 566 (1979)
[3] *Van Dyke v. Illinois Commercial Men's Ass'n*, 358 Ill 458 (1934)

case, the state court is reinvested with jurisdiction. The *Van Dyke* case derived its decision from the United States Supreme Court decisions which held that actions return to the state court upon remandment.

Considering the foregoing cases, along with the fact that it is well settled that public documents which are included in the records of other courts may be the subject of judicial notice, along with the fact the parties handed the Clay County Court a copy of the Order of Remand which was filed *instanter* on July 2, 2020, this Court finds that the Clay County Circuit Court did in fact have jurisdiction.

IT IS HEREBY ORDERED:

A. The Governor's Motion To Vacate the July 2, 2020 Order for lack of jurisdiction is denied;

B. If the Governor still wishes to have this Court reconsider the Clay County Order of July 2, 2020 granting summary judgment on counts II and III due to errors in applying existing law, any such motion shall be filed by October 30, 2020;

C. Any response to the Motion to Reconsider the granting of summary judgment on counts II and III must be filed by November 12, 2020;

D. Any Reply in Support of the Motion to Reconsider the granting of summary judgment on counts II and III should be filed by November 20, 2020;

E. In order for the Court to consider all pleadings, any written pleading must be emailed to the Court by the prescribed deadlines, but no later than Monday, November 30, 2020.

F. Oral arguments for the Motion to Reconsider the granting of summary judgment is set for **Monday, December 7, 2020 at 1:30 p.m.** All attorneys are to appear in person.

ENTERED: October 19, 2020

Raylene D. Grischow

Raylene Grischow, *Circuit Court Judge*

The NEW ENGLAND JOURNAL of MEDICINE

May 2020
Harvard Univ.

Perspective

MAY 21, 2020

Universal Masking in Hospitals in the Covid-19 Era

Michael Klompas, M.D., M.P.H., Charles A. Morris, M.D., M.P.H., Julia Sinclair, M.B.A., Madelyn Pearson, D.N.P., R.N., and Erica S. Shenoy, M.D., Ph.D.

plannedemic

As the SARS-CoV-2 pandemic continues to explode, hospital systems are scrambling to intensify their measures for protecting patients and health care workers from the virus. An increasing number of frontline providers are wondering whether this effort should include universal use of masks by all health care workers. Universal masking is already standard practice in Hong Kong, Singapore, and other parts of Asia and has recently been adopted by a handful of U.S. hospitals.

We know that wearing a mask outside health care facilities offers little, if any, protection from infection. Public health authorities define a significant exposure to Covid-19 as face-to-face contact within 6 feet with a patient with symptomatic Covid-19 that is sustained for at least a few minutes (and some say more than 10 minutes or even 30 minutes). The chance of catching Covid-19 from a passing interaction in a public space is therefore minimal. In many cases, the desire for widespread masking is a reflexive reaction to anxiety over the pandemic.

The calculus may be different, however, in health care settings. First and foremost, a mask is a core component of the personal protective equipment (PPE) clinicians need when caring for symptomatic patients with respiratory viral infections, in conjunction with gown, gloves, and eye protection. Masking in this context is already part of routine operations for most hospitals. What is less clear is whether a mask offers any further protection in health care settings in which the wearer has no direct interactions with symptomatic patients. There are two scenarios in which there may be possible benefits.

The first is during the care of a patient with unrecognized Covid-19. A mask alone in this setting will reduce risk only slightly, however, since it does not provide protection from droplets that may enter the eyes or from fomites on the patient or in the environment that providers may pick up on their hands and carry to their mucous membranes (particularly given the concern that mask wearers may have an increased tendency to touch their faces).

More compelling is the possibility that wearing a mask may reduce the likelihood of transmission from asymptomatic and minimally symptomatic health care workers with Covid-19 to other providers and patients. This concern increases as Covid-19 becomes more widespread in the community. We face a constant risk that a health care worker with

early infection may bring the virus into our facilities and transmit it to others. Transmission from people with asymptomatic infection has been well documented, although it is unclear to what extent such transmission contributes to the overall spread of infection.[1-3]

More insidious may be the health care worker who comes to work with mild and ambiguous symptoms, such as fatigue or muscle aches, or a scratchy throat and mild nasal congestion, that they attribute to working long hours or stress or seasonal allergies, rather than recognizing that they may have early or mild Covid-19. In our hospitals, we have already seen a number of instances in which staff members either came to work well but developed symptoms of Covid-19 partway through their shifts or worked with mild and ambiguous symptoms that were subsequently diagnosed as Covid-19. These cases have led to large numbers of our patients and staff members being exposed to the virus and a handful of potentially linked infections in health care workers. Masking all providers might limit transmission from these sources by stopping asymptomatic and minimally symptomatic health care workers from spreading virus-laden oral and nasal droplets.

What is clear, however, is that universal masking alone is not a panacea. A mask will not protect providers caring for a patient with active Covid-19 if it's not accompanied by meticulous hand hygiene, eye protection, gloves, and a gown. A mask alone will not prevent health care workers with early Covid-19 from contaminating their hands and spreading the virus to patients and colleagues. Focusing on universal masking alone may, paradoxically, lead to more transmission of Covid-19 if it diverts attention from implementing more fundamental infection-control measures.

Such measures include vigorous screening of all patients coming to a facility for symptoms of Covid-19 and immediately getting them masked and into a room; early implementation of contact and droplet precautions, including eye protection, for all symptomatic patients and erring on the side of caution when in doubt; rescreening all admitted patients daily for signs and symptoms of Covid-19 in case an infection was incubating on admission or they were exposed to the virus in the hospital; having a low threshold for testing patients with even mild symptoms potentially attributable to a viral respiratory infection (this includes patients with pneumonia, given that a third or more of pneumonias are caused by viruses rather than bacteria); requiring employees to attest that they have no symptoms before starting work each day; being attentive to physical distancing between staff members in all settings (including potentially neglected settings such as elevators, hospital shuttle buses, clinical rounds, and work rooms); restricting and screening visitors; and increasing the frequency and reliability of hand hygiene.

The extent of marginal benefit of universal masking over and above these foundational measures is debatable. It depends on the prevalence of health care workers with asymptomatic and minimally symptomatic infections as well as the relative contribution of this population to the spread of infection. It is informative, in this regard, that the prevalence of Covid-19 among asymptomatic evacuees from Wuhan during the height of the epidemic there was only 1 to 3%.[4,5] Modelers assessing the spread of infection in Wuhan have noted the importance of undiagnosed infections in fueling the spread of Covid-19 while also acknowledging that the transmission risk from this population is likely to be lower than the risk of spread from symptomatic patients.[3] And then the potential benefits of universal masking need to be balanced against the future risk of running out of masks and thereby exposing clinicians to the much greater risk of caring for symptomatic patients without a mask. Providing each health care worker with one mask per day for extended use, however, may paradoxically improve inventory control by reducing one-time uses and facilitating centralized workflows for allocating masks without risk assessments at the individual-employee level.

There may be additional benefits to broad masking policies that extend beyond their technical contribution to reducing pathogen transmission. Masks are visible reminders of an otherwise invisible yet widely prevalent pathogen and may remind people of the importance of social distancing and other infection-control measures.

It is also clear that masks serve symbolic roles. Masks are not only tools, they are also talismans that may help increase health care workers' perceived sense of safety, well-being, and trust in their hospitals. Although such reactions may not be strictly logical, we are all subject to fear and anxiety, especially during times of crisis. One might argue that fear and anxiety are better countered with data and education than with a marginally beneficial mask, par-

What a bunch of baloney This is planned all to control the people. It has been working for more than a year.

ticularly in light of the worldwide mask shortage, but it is difficult to get clinicians to hear this message in the heat of the current crisis. Expanded masking protocols' greatest contribution may be to reduce the transmission of anxiety, over and above whatever role they may play in reducing transmission of Covid-19. The potential value of universal masking in giving health care workers the confidence to absorb and implement the more foundational infection-prevention practices described above may be its greatest contribution.

Disclosure forms provided by the authors are available at NEJM.org.

From the Department of Population Medicine, Harvard Medical School and Harvard Pilgrim Health Care Institute (M.K.), Brigham and Women's Hospital (M.K., C.A.M., J.S., M.P.), Harvard Medical School (M.K., C.A.M., E.S.S.), and the Infection Control Unit and Division of Infectious Diseases, Massachusetts General Hospital (E.S.S.) — all in Boston.

This article was published on April 1, 2020, at NEJM.org.

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DOI: 10.1056/NEJMp2006372

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Facemasks in the COVID-19 era: A health hypothesis

Baruch Vainshelboim *

Stanford Univ. Nov 2020

Cardiology Division, Veterans Affairs Palo Alto Health Care System/Stanford University, Palo Alto, CA, United States

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Safety
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ABSTRACT

Many countries across the globe utilized medical and non-medical facemasks as non-pharmaceutical intervention for reducing the transmission and infectivity of coronavirus disease-2019 (COVID-19). Although, scientific evidence supporting facemasks' efficacy is lacking, adverse physiological, psychological and health effects are established. Is has been hypothesized that facemasks have compromised safety and efficacy profile and should be avoided from use. The current article comprehensively summarizes scientific evidences with respect to wearing facemasks in the COVID-19 era, providing prosper information for public health and decisions making.

Introduction

Facemasks are part of non-pharmaceutical interventions providing some breathing barrier to the mouth and nose that have been utilized for reducing the transmission of respiratory pathogens [1]. Facemasks can be medical and non-medical, where two types of the medical masks primarily used by healthcare workers [1,2]. The first type is National Institute for Occupational Safety and Health (NIOSH)-certified N95 mask, a filtering face-piece respirator, and the second type is a surgical mask [1]. The designed and intended uses of N95 and surgical masks are different in the type of protection they potentially provide. The N95s are typically composed of electret filter media and seal tightly to the face of the wearer, whereas surgical masks are generally loose fitting and may or may not contain electret-filtering media. The N95s are designed to reduce the wearer's inhalation exposure to infectious and harmful particles from the environment such as during extermination of insects. In contrast, surgical masks are designed to provide a barrier protection against splash, spittle and other body fluids to spray from the wearer (such as surgeon) to the sterile environment (patient during operation) for reducing the risk of contamination [1].

The third type of facemasks are the non-medical cloth or fabric masks. The non-medical facemasks are made from a variety of woven and non-woven materials such as Polypropylene, Cotton, Polyester, Cellulose, Gauze and Silk. Although non-medical cloth or fabric facemasks are neither a medical device nor personal protective equipment, some standards have been developed by the French Standardization Association (AFNOR Group) to define a minimum performance for filtration and breathability capacity [2]. The current article reviews the scientific evidences with respect to safety and efficacy of wearing facemasks, describing the physiological and psychological effects and the potential long-term consequences on health.

Hypothesis

On January 30, 2020, the World Health Organization (WHO) announced a global public health emergency of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) causing illness of coronavirus disease-2019 (COVID-19) [3]. As of October 1, 2020, worldwide 34,166,633 cases were reported and 1,018,876 have died with virus diagnosis. Interestingly, 99% of the detected cases with SARS-CoV-2 are asymptomatic or have mild condition, which contradicts with the virus name (*severe* acute respiratory syndrome-coronavirus-2) [4]. Although infection fatality rate (number of death cases divided by number of reported cases) initially seems quite high 0.029 (2.9%) [4], this overestimation related to limited number of COVID-19 tests performed which biases towards higher rates. Given the fact that asymptomatic or minimally symptomatic cases is several times higher than the number of reported cases, the case fatality rate is considerably less than 1% [5]. This was confirmed by the head of National Institute of Allergy and Infectious Diseases from US stating, "the overall clinical consequences of COVID-19 are similar to those of severe seasonal influenza" [5], having a case fatality rate of approximately 0.1% [5–8]. In addition, data from hospitalized patients with COVID-19 and general public indicate that the majority of deaths were among older and chronically ill individuals, supporting the possibility that the virus may exacerbates existing conditions but rarely causes death by itself [9,10]. SARS-CoV-2 primarily

* Address: VA Palo Alto Health Care System, Cardiology 111C, 3801 Miranda Ave, Palo Alto, CA 94304, United States.
E-mail address: baruch.v1981@gmail.com.

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affects respiratory system and can cause complications such as acute respiratory distress syndrome (ARDS), respiratory failure and death [3,9]. It is not clear however, what the scientific and clinical basis for wearing facemasks as protective strategy, given the fact that facemasks restrict breathing, causing hypoxemia and hypercapnia and increase the risk for respiratory complications, self-contamination and exacerbation of existing chronic conditions [2,11–14].

Of note, hyperoxia or oxygen supplementation (breathing air with high partial O_2 pressures that above the sea levels) has been well established as therapeutic and curative practice for variety acute and chronic conditions including respiratory complications [11,15]. It fact, the current standard of care practice for treating hospitalized patients with COVID-19 is breathing 100% oxygen [16–18]. Although several countries mandated wearing facemask in health care settings and public areas, scientific evidences are lacking supporting their efficacy for reducing morbidity or mortality associated with infectious or viral diseases [2,14,19]. Therefore, it has been hypothesized: 1) the practice of wearing facemasks has compromised safety and efficacy profile, 2) Both medical and non-medical facemasks are ineffective to reduce human-to-human transmission and infectivity of SARS-CoV-2 and COVID-19, 3) Wearing facemasks has adverse physiological and psychological effects, 4) Long-term consequences of wearing facemasks on health are detrimental.

Evolution of hypothesis

Breathing Physiology

Breathing is one of the most important physiological functions to sustain life and health. Human body requires a continuous and adequate oxygen (O_2) supply to all organs and cells for normal function and survival. Breathing is also an essential process for removing metabolic byproducts [carbon dioxide (CO_2)] occurring during cell respiration [12,13]. It is well established that acute significant deficit in O_2 (hypoxemia) and increased levels of CO_2 (hypercapnia) even for few minutes can be severely harmful and lethal, while chronic hypoxemia and hypercapnia cause health deterioration, exacerbation of existing conditions, morbidity and ultimately mortality [11,20–22]. Emergency medicine demonstrates that 5–6 min of severe hypoxemia during cardiac arrest will cause brain death with extremely poor survival rates [20–23]. On the other hand, chronic mild or moderate hypoxemia and hypercapnia such as from wearing facemasks resulting in shifting to higher contribution of anaerobic energy metabolism, decrease in pH levels and increase in cells and blood acidity, toxicity, oxidative stress, chronic inflammation, immunosuppression and health deterioration [11–13,24].

Efficacy of facemasks

The physical properties of medical and non-medical facemasks suggest that facemasks are ineffective to block viral particles due to their difference in scales [16,17,25]. According to the current knowledge, the virus SARS-CoV-2 has a diameter of 60 nm to 140 nm [nanometers (billionth of a meter)] [16,17], while medical and non-medical facemasks' thread diameter ranges from 55 μm to 440 μm [micrometers (one millionth of a meter), which is more than 1000 times larger [25]. Due to the difference in sizes between SARS-CoV-2 diameter and facemasks thread diameter (the virus is 1000 times smaller), SARS-CoV-2 can easily pass through any facemask [25]. In addition, the efficiency filtration rate of facemasks is poor, ranging from 0.7% in non-surgical, cotton-gauze woven mask to 26% in cotton sweeter material [2]. With respect to surgical and N95 medical facemasks, the efficiency filtration rate falls to 15% and 58%, respectively when even small gap between the mask and the face exists [25].

Clinical scientific evidence challenges further the efficacy of facemasks to block human-to-human transmission or infectivity. A randomized controlled trial (RCT) of 246 participants [123 (50%) symptomatic)] who were allocated to either wearing or not wearing surgical facemask, assessing viruses transmission including coronavirus [26]. The results of this study showed that among symptomatic individuals (those with fever, cough, sore throat, runny nose ect…) there was no difference between wearing and not wearing facemask for coronavirus droplets transmission of particles of >5 μm. Among asymptomatic individuals, there was no droplets or aerosols coronavirus detected from any participant with or without the mask, suggesting that asymptomatic individuals do not transmit or infect other people [26]. This was further supported by a study on infectivity where 445 asymptomatic individuals were exposed to asymptomatic SARS-CoV-2 carrier (been positive for SARS-CoV-2) using close contact (shared quarantine space) for a median of 4 to 5 days. The study found that none of the 445 individuals was infected with SARS-CoV-2 confirmed by real-time reverse transcription polymerase [27].

A *meta*-analysis among health care workers found that compared to no masks, surgical mask and N95 respirators were not effective against transmission of viral infections or influenza-like illness based on six RCTs [28]. Using separate analysis of 23 observational studies, this *meta*-analysis found no protective effect of medical mask or N95 respirators against SARS virus [28]. A recent systematic review of 39 studies including 33,867 participants in community settings (self-report illness), found no difference between N95 respirators versus surgical masks and surgical mask versus no masks in the risk for developing influenza or influenza-like illness, suggesting their ineffectiveness of blocking viral transmissions in community settings [29].

Another *meta*-analysis of 44 non-RCT studies (n = 25,697 participants) examining the potential risk reduction of facemasks against SARS, middle east respiratory syndrome (MERS) and COVID-19 transmissions [30]. The *meta*-analysis included four specific studies on COVID-19 transmission (5,929 participants, primarily health-care workers used N95 masks). Although the overall findings showed reduced risk of virus transmission with facemasks, the analysis had severe limitations to draw conclusions. One of the four COVID-19 studies had zero infected cases in both arms, and was excluded from *meta*-analytic calculation. Other two COVID-19 studies had unadjusted models, and were also excluded from the overall analysis. The *meta*-analytic results were based on only one COVID-19, one MERS and 8 SARS studies, resulting in high selection bias of the studies and contamination of the results between different viruses. Based on four COVID-19 studies, the *meta*-analysis failed to demonstrate risk reduction of facemasks for COVID-19 transmission, where the authors reported that the results of *meta*-analysis have low certainty and are inconclusive [30].

In early publication the WHO stated that "facemasks are not required, as no evidence is available on its usefulness to protect non-sick persons" [14]. In the same publication, the WHO declared that "cloth (e.g. cotton or gauze) masks are not recommended under any circumstance" [14]. Conversely, in later publication the WHO stated that the usage of fabric-made facemasks (Polypropylene, Cotton, Polyester, Cellulose, Gauze and Silk) is a general community practice for "preventing the infected wearer transmitting the virus to others and/or to offer protection to the healthy wearer against infection (prevention)" [2]. The same publication further conflicted itself by stating that due to the lower filtration, breathability and overall performance of fabric facemasks, the usage of woven fabric mask such as cloth, and/or non-woven fabrics, should only be considered for infected persons and not for prevention practice in asymptomatic individuals [2]. The Central for Disease Control and Prevention (CDC) made similar recommendation, stating that only symptomatic persons should consider wearing facemask, while for asymptomatic individuals this practice is not recommended [31]. Consistent with the CDC, clinical scientists from Departments of Infectious Diseases and Microbiology in Australia counsel against facemasks usage for health-care workers, arguing that there is no justification for such practice while normal caring relationship between patients and medical staff could be compromised [32].

Moreover, the WHO repeatedly announced that "at present, there is no direct evidence (from studies on COVID-19) on the effectiveness face masking of healthy people in the community to prevent infection of respiratory viruses, including COVID-19"[2]. Despite these controversies, the potential harms and risks of wearing facemasks were clearly acknowledged. These including self-contamination due to hand practice or non-replaced when the mask is wet, soiled or damaged, development of facial skin lesions, irritant dermatitis or worsening acne and psychological discomfort. Vulnerable populations such as people with mental health disorders, developmental disabilities, hearing problems, those living in hot and humid environments, children and patients with respiratory conditions are at significant health risk for complications and harm [2].

Physiological effects of wearing facemasks

Wearing facemask mechanically restricts breathing by increasing the resistance of air movement during both inhalation and exhalation process [12,13]. Although, intermittent (several times a week) and repetitive (10–15 breaths for 2–4 sets) increase in respiration resistance may be adaptive for strengthening respiratory muscles [33,34], prolonged and continues effect of wearing facemask is maladaptive and could be detrimental for health [11–13]. In normal conditions at the sea level, air contains 20.93% O_2 and 0.03% CO_2, providing partial pressures of 100 mmHg and 40 mmHg for these gases in the arterial blood, respectively. These gas concentrations significantly altered when breathing occurs through facemask. A trapped air remaining between the mouth, nose and the facemask is rebreathed repeatedly in and out of the body, containing low O_2 and high CO_2 concentrations, causing hypoxemia and hypercapnia [11–13,35,36]. Severe hypoxemia may also provoke cardiopulmonary and neurological complications and is considered an important clinical sign in cardiopulmonary medicine [37–42]. Low oxygen content in the arterial blood can cause myocardial ischemia, serious arrhythmias, right or left ventricular dysfunction, dizziness, hypotension, syncope and pulmonary hypertension [43]. Chronic low-grade hypoxemia and hypercapnia as result of using facemask can cause exacerbation of existing cardiopulmonary, metabolic, vascular and neurological conditions [37–42]. Table 1 summarizes the physiological, psychological effects of wearing facemask and their potential long-term consequences for health.

In addition to hypoxia and hypercapnia, breathing through facemask residues bacterial and germs components on the inner and outside layer of the facemask. These toxic components are repeatedly rebreathed back into the body, causing self-contamination. Breathing through facemasks also increases temperature and humidity in the space between the mouth and the mask, resulting a release of toxic particles from the mask's materials [1,2,19,26,35,36]. A systematic literature review estimated that aerosol contamination levels of facemasks including 13 to 202,549 different viruses [1]. Rebreathing contaminated air with high bacterial and toxic particle concentrations along with low O_2 and high CO_2 levels continuously challenge the body homeostasis, causing self-toxicity and immunosuppression [1,2,19,26,35,36].

Table 1
Physiological and Psychological Effects of Wearing Facemask and Their Potential Health Consequences.

Physiological Effects	Psychological Effect	Health Consequences
• Hypoxemia • Hypercapnia • Shortness of breath • Increase lactate concentration • Decline in pH levels • Acidosis • Toxicity • Inflammation • Self-contamination • Increase in stress hormones level (adrenaline, noradrenaline and cortisol) • Increased muscle tension • Immunosuppression	• Activation of "fight or flight" stress response • Chronic stress condition • Fear • Mood disturbances • Insomnia • Fatigue • Compromised cognitive performance	• Increased predisposition for viral and infection illnesses • Headaches • Anxiety • Depression • Hypertension • Cardiovascular disease • Cancer • Diabetes • Alzheimer disease • Exacerbation of existing conditions and diseases • Accelerated aging process • Health deterioration • Premature mortality

A study on 39 patients with renal disease found that wearing N95 facemask during hemodialysis significantly reduced arterial partial oxygen pressure (from PaO_2 101.7 to 92.7 mm Hg), increased respiratory rate (from 16.8 to 18.8 breaths/min), and increased the occurrence of chest discomfort and respiratory distress [35]. Respiratory Protection Standards from Occupational Safety and Health Administration, US Department of Labor states that breathing air with O_2 concentration below 19.5% is considered oxygen-deficiency, causing physiological and health adverse effects. These include increased breathing frequency, accelerated heartrate and cognitive impairments related to thinking and coordination [36]. A chronic state of mild hypoxia and hypercapnia has been shown as primarily mechanism for developing cognitive dysfunction based on animal studies and studies in patients with chronic obstructive pulmonary disease [44].

The adverse physiological effects were confirmed in a study of 53 surgeons where surgical facemask were used during a major operation. After 60 min of facemask wearing the oxygen saturation dropped by more than 1% and heart rate increased by approximately five beats/min [45]. Another study among 158 health-care workers using protective personal equipment primarily N95 facemasks reported that 81% (128 workers) developed new headaches during their work shifts as these become mandatory due to COVID-19 outbreak. For those who used the N95 facemask greater than 4 h per day, the likelihood for developing a headache during the work shift was approximately four times higher [Odds ratio = 3.91, 95% CI (1.35–11.31) p = 0.012], while 82.2% of the N95 wearers developed the headache already within ≤10 to 50 min [46].

With respect to cloth facemask, a RCT using four weeks follow up compared the effect of cloth facemask to medical masks and to no masks on the incidence of clinical respiratory illness, influenza-like illness and laboratory-confirmed respiratory virus infections among 1607 participants from 14 hospitals [19]. The results showed that there were no difference between wearing cloth masks, medical masks and no masks for incidence of clinical respiratory illness and laboratory-confirmed respiratory virus infections. However, a large harmful effect with more than 13 times higher risk [Relative Risk = 13.25 95% CI (1.74 to 100.97) was observed for influenza-like illness among those who were wearing cloth masks [19]. The study concluded that cloth masks have significant health and safety issues including moisture retention, reuse, poor filtration and increased risk for infection, providing recommendation against the use of cloth masks [19].

Psychological effects of wearing facemasks

Psychologically, wearing facemask fundamentally has negative effects on the wearer and the nearby person. Basic human-to-human connectivity through face expression is compromised and self-identity is somewhat eliminated [47–49]. These dehumanizing movements partially delete the uniqueness and individuality of person who wearing the facemask as well as the connected person [49]. Social connections and relationships are basic human needs, which innately inherited in all people, whereas reduced human-to-human connections are associated with poor mental and physical health [50,51]. Despite escalation in technology and globalization that would presumably foster social connections, scientific findings show that people are becoming increasingly more socially isolated, and the prevalence of loneliness is increasing in last few decades [50,52]. Poor social connections are closely related to

isolation and loneliness, considered significant health related risk factors [50–53].

A *meta*-analysis of 91 studies of about 400,000 people showed a 13% increased morality risk among people with low compare to high contact frequency [53]. Another *meta*-analysis of 148 prospective studies (308,849 participants) found that poor social relationships was associated with 50% increased mortality risk. People who were socially isolated or fell lonely had 45% and 40% increased mortality risk, respectively. These findings were consistent across ages, sex, initial health status, cause of death and follow-up periods [52]. Importantly, the increased risk for mortality was found comparable to smoking and exceeding well-established risk factors such as obesity and physical inactivity [52]. An umbrella review of 40 systematic reviews including 10 *meta*-analyses demonstrated that compromised social relationships were associated with increased risk of all-cause mortality, depression, anxiety suicide, cancer and overall physical illness [51].

As described earlier, wearing facemasks causing hypoxic and hypercapnic state that constantly challenges the normal homeostasis, and activates "fight or flight" stress response, an important survival mechanism in the human body [11–13]. The acute stress response includes activation of nervous, endocrine, cardiovascular, and the immune systems [47,54–56]. These include activation of the limbic part of the brain, release stress hormones (adrenalin, neuro-adrenalin and cortisol), changes in blood flow distribution (vasodilation of peripheral blood vessels and vasoconstriction of visceral blood vessels) and activation of the immune system response (secretion of macrophages and natural killer cells) [47,48]. Encountering people who wearing facemasks activates innate stress-fear emotion, which is fundamental to all humans in danger or life threating situations, such as death or unknown, unpredictable outcome. While acute stress response (seconds to minutes) is adaptive reaction to challenges and part of the survival mechanism, chronic and prolonged state of stress-fear is maladaptive and has detrimental effects on physical and mental health. The repeatedly or continuously activated stress-fear response causes the body to operate on survival mode, having sustain increase in blood pressure, pro-inflammatory state and immunosuppression [47,48].

Long-Term health consequences of wearing facemasks

Long-term practice of wearing facemasks has strong potential for devastating health consequences. Prolonged hypoxic-hypercapnic state compromises normal physiological and psychological balance, deteriorating health and promotes the developing and progression of existing chronic diseases [11–13,23,38,39,43,47,48,57]. For instance, ischemic heart disease caused by hypoxic damage to the myocardium is the most common form of cardiovascular disease and is a number one cause of death worldwide (44% of all non-communicable diseases) with 17.9 million deaths occurred in 2016 [57]. Hypoxia also playing an important role in cancer burden [58]. Cellular hypoxia has strong mechanistic feature in promoting cancer initiation, progression, metastasis, predicting clinical outcomes and usually presents a poorer survival in patients with cancer. Most solid tumors present some degree of hypoxia, which is independent predictor of more aggressive disease, resistance to cancer therapies and poorer clinical outcomes [59,60]. Worth note, cancer is one of the leading causes of death worldwide, with an estimate of more than 18 million new diagnosed cases and 9.6 million cancer-related deaths occurred in 2018 [61].

With respect to mental health, global estimates showing that COVID-19 will cause a catastrophe due to collateral psychological damage such as quarantine, lockdowns, unemployment, economic collapse, social isolation, violence and suicides [62–64]. Chronic stress along with hypoxic and hypercapnic conditions knocks the body out of balance, and can cause headaches, fatigue, stomach issues, muscle tension, mood disturbances, insomnia and accelerated aging [47,48,65–67]. This state suppressing the immune system to protect the body from viruses and bacteria, decreasing cognitive function, promoting the developing and exacerbating the major health issues including hypertension, cardiovascular disease, diabetes, cancer, Alzheimer disease, rising anxiety and depression states, causes social isolation and loneliness and increasing the risk for prematurely mortality [47,48,51,56,66].

Conclusion

The existing scientific evidences challenge the safety and efficacy of wearing facemask as preventive intervention for COVID-19. The data suggest that both medical and non-medical facemasks are ineffective to block human-to-human transmission of viral and infectious disease such SARS-CoV-2 and COVID-19, supporting against the usage of facemasks. Wearing facemasks has been demonstrated to have substantial adverse physiological and psychological effects. These include hypoxia, hypercapnia, shortness of breath, increased acidity and toxicity, activation of fear and stress response, rise in stress hormones, immunosuppression, fatigue, headaches, decline in cognitive performance, predisposition for viral and infectious illnesses, chronic stress, anxiety and depression. Long-term consequences of wearing facemask can cause health deterioration, developing and progression of chronic diseases and premature death. Governments, policy makers and health organizations should utilize prosper and scientific evidence-based approach with respect to wearing facemasks, when the latter is considered as preventive intervention for public health.

CRediT authorship contribution statement

Baruch Vainshelboim: Conceptualization, Data curation, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Predominant Role of Bacterial Pneumonia as a Cause of Death in Pandemic Influenza: Implications for Pandemic Influenza Preparedness

David M. Morens, Jeffery K. Taubenberger, and Anthony S. Fauci

National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

(See the editorial commentary by McCullers, on pages XXX–XXX.)

Background. Despite the availability of published data on 4 pandemics that have occurred over the past 120 years, there is little modern information on the causes of death associated with influenza pandemics.

Methods. We examined relevant information from the most recent influenza pandemic that occurred during the era prior to the use of antibiotics, the 1918–1919 "Spanish flu" pandemic. We examined lung tissue sections obtained during 58 autopsies and reviewed pathologic and bacteriologic data from 109 published autopsy series that described 8398 individual autopsy investigations.

Results. The postmortem samples we examined from people who died of influenza during 1918–1919 uniformly exhibited severe changes indicative of bacterial pneumonia. Bacteriologic and histopathologic results from published autopsy series clearly and consistently implicated secondary bacterial pneumonia caused by common upper respiratory–tract bacteria in most influenza fatalities.

Conclusions. The majority of deaths in the 1918–1919 influenza pandemic likely resulted directly from secondary bacterial pneumonia caused by common upper respiratory–tract bacteria. Less substantial data from the subsequent 1957 and 1968 pandemics are consistent with these findings. If severe pandemic influenza is largely a problem of viral-bacterial copathogenesis, pandemic planning needs to go beyond addressing the viral cause alone (e.g., influenza vaccines and antiviral drugs). Prevention, diagnosis, prophylaxis, and treatment of secondary bacterial pneumonia, as well as stockpiling of antibiotics and bacterial vaccines, should also be high priorities for pandemic planning.

Masks the vector

"If grippe condemns, the secondary infections execute" [1, p. 448].

——Louis Cruveilhier, 1919

Influenza pandemic preparedness strategies in the United States [2] assume 3 levels of potential severity corresponding to the 20th century pandemics of H1N1 "Spanish flu" (1918–1919), H2N2 "Asian flu" (1957–1958), and H3N2 "Hong Kong flu" (1968–1969), which were responsible for an estimated 675,000 [3], 86,000 [4], and 56,300 [5] excess deaths in the United States, respectively. Extrapolation from 1918–1919 pandemic data to the current population and age profile has led United States government officials to plan for more than 1.9 million excess deaths during a severe pandemic [2].

An important question related to pandemic preparedness remains unanswered: what killed people during the 1918–1919 pandemic and subsequent influenza pandemics? In the present study, we have examined recut tissue specimens obtained during autopsy from 58 influenza victims in 1918–1919, and have reviewed epidemiologic, pathologic, and microbiologic data from published reports for 8398 postmortem examinations bearing on this question. We have also reviewed relevant information, accumulated over 9 decades, related to the circulation of descendants of the 1918 virus. With the recent reconstruction of the 1918 pandemic influenza virus, investigators have begun to examine why it was so highly fatal [6, 7]. Based on contemporary and modern evidence, we conclude here that influenza A virus infec-

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Reprints or correspondence: David M. Morens, MD, Bldg. 31, Room 7A-10, 31 Center Dr., MSC 2520, National Institute of Allergy and Inectious Diseases, National Institutes of Health, Bethesda, MD 20892–2520 (dmorens@niaid.nih.gov).

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tion in conjunction with bacterial infection led to most of the deaths during the 1918–1919 pandemic.

METHODS

Examination of tissue specimens from 1918–1919 influenza fatalities. We reviewed hematoxylin and eosin–stained slides recut from blocks of lung tissue obtained during autopsy from 58 influenza fatalities in 1918–1919. These materials, sent during the pandemic from various United States military bases to the National Tissue Repository of the Armed Forces Institute of Pathology [8–10], represent all known influenza cases from this collection for which lung tissue is available.

Pathology and bacteriology research records from the 1918–1919 influenza pandemic. We reviewed the late 19th- and early 20th-century literature on gross and microscopic influenza pathology and bacteriology, including evidence from 1918–1919 autopsy series with postmortem cultures of lung tissue, blood samples (usually heart blood), pleural fluid, and samples from other compartments. In an effort to obtain all publications possibly reporting influenza pathology and/or bacteriology in 1918–1919, we searched major bibliographic sources [e.g., 11–17] for papers in all languages and tables of contents of major journals in English, German, and French; in addition, we searched all of the papers we identified for additional citations. From more than 2000 such publications, we carefully examined the 1539 reports that contained human pathologic and/or bacteriologic findings (the full bibliographic list available at http://www3.niaid.nih.gov/topics/Flu/1918/bibliography.htm), 109 of which provided useful bacteriologic information derived from 173 autopsy series. These series reported 8398 individual autopsy investigations undertaken in 15 countries, which can be characterized as follows: 96 postmortem lung tissue culture series, 42 blood culture series, and 35 pleural fluid culture series. When they were published as parts of an autopsy series, we included in our analyses antemortem cultures of blood and pleural fluid samples, which were mostly obtained during the terminal stages of illness. A priori, we stratified data by military and civilian populations (see Discussion), and by the quality of lung tissue culture results, considering to be of "higher quality" the 68 autopsy series with lung tissue culture results that reported, for all autopsies, both the presence and absence of negative culture results and the bacterial components of mixed culture results.

RESULTS

Background epidemiologic data on influenza mortality rates in 1918–1919. Although death certificates listing cardiac and other chronic causes of death increased in number during the time frame of the 1918–1919 pandemic [18], for all age groups death was predominantly associated with pneumonia and related pulmonary complications [13, 14, 18–20]. The pandemic caused a "W-shaped" age-specific mortality curve, which exhibited peaks in infancy, between about 20–40 years of age, and in elderly individuals [3, 21]. In all age groups younger than ~65 years, the influenza mortality rate was elevated beyond what would have been expected on the basis of data from the previous pandemic of "Russian influenza" (1889–1893) [3, 22, 23]. The increased fatality rate in the 3 high-risk age groups was predominantly due to the increased frequency of bronchopneumonia, not to increased incidence of influenza or an increased bronchopneumonia case-fatality rate [19]. Because few autopsy reports and, to our knowledge, no autopsy series addressed conditions other than predominantly pulmonary complications, nonpulmonary causes of death are not considered here.

Histologic examination of lung tissue from 1918 victims. The examination of recut lung tissue sections from 1918–1919 influenza case material revealed, in virtually all cases, compelling histologic evidence of severe acute bacterial pneumonia, either as the predominant pathology or in conjunction with underlying pathologic features now believed to be associated with influenza virus infection [10, 24] (figure 1). The latter include necrosis and desquamation of the respiratory epithelium of the tracheobronchial and bronchiolar tree, dilation of alveolar ducts, hyaline membranes, and evidence of bronchial and/or bronchiolar epithelial repair [25, 26]. The majority of the cases examined demonstrated asynchronous histopathological changes, in which the various stages of development of the infectious process, from early bronchiolar changes to severe bacterial parenchymal destruction, were noted in focal areas. The histologic spectrum observed in the cases corresponded to the characteristic pathology of bacterial pneumonia, including bronchopneumonia [10,24–33]: lobar consolidation with pulmonary infiltration by neutrophils in pneumococcal pneumonia; a bronchopneumonic pattern, edema, and pleural effusions in streptococcal and sometimes in pneumococcal pneumonia; and in staphylococcal pneumonia, multiple small abscesses with a marked neutrophilic infiltration in airways and alveoli [27]. Bacteria were commonly observed in the sections, often in massive numbers.

Published pathologic and/or bacteriologic findings from the 1918–1919 influenza pandemic. Although the cause of influenza was disputed in 1918, there was almost universal agreement among experts [e.g., 20, 27–33] that deaths were virtually never caused by the unidentified etiologic agent itself, but resulted directly from severe secondary pneumonia caused by well-known bacterial "pneumopathogens" that colonized the upper respiratory tract (predominantly pneumococci, streptococci, and staphylococci). Without this secondary bacterial pneumonia, experts generally believed that most patients would have recovered [20]. In type, pattern, and case-fatality rate, influenza-associated bacterial pneumonia was typical of pneumonia that was endemic during periods when influenza was not prevalent [25, 28, 33, 34]. As described above, in cases for which a single lung pathogen was recovered from culture, the anatomical-pathological type of the pneumonia usually corresponded to what was expected. Bacteria were commonly observed in cases of pneumonia caused by each of these pathogens. Such findings

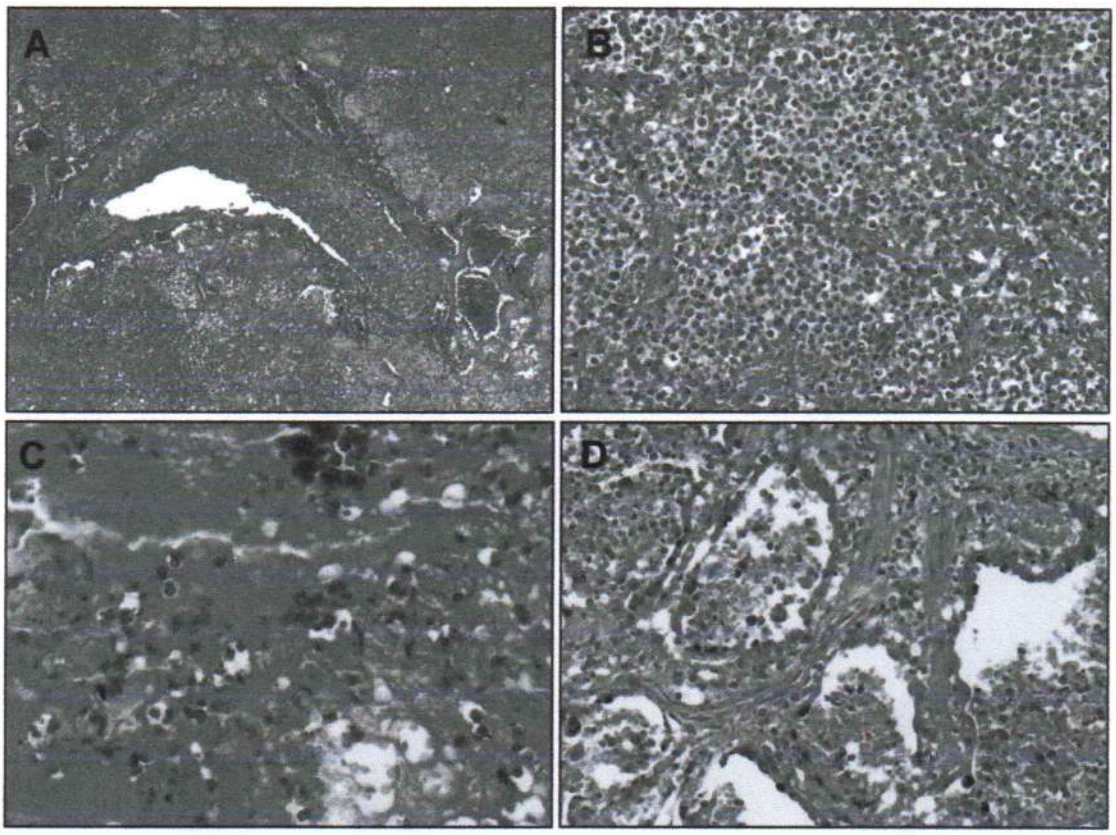

Figure 1. Examples of hematoxylin and eosin–stained postmortem lung sections from 4 victims of the 1918–1919 influenza pandemic (see text). *A*, Typical picture of severe, widespread bacterial bronchopneumonia with transmural infiltration of neutrophils in a bronchiole and with neutrophils filling the airspaces of surrounding alveoli (original magnification, 40×). *B*, Massive infiltration of neutrophils in the airspaces of alveoli associated with bacterial bronchopneumonia as in A (original magnification, 200×). *C*, Bronchopneumonia with intra-alveolar edema and hemorrhage. Numerous bacteria are visible both in the edema fluid and in the cytoplasm of macrophages (original magnification, 400×). *D*, Bronchopneumonia with evidence of pulmonary repair. The alveolar epithelium is hyperplastic; interstitial fibrosis is seen between alveoli (original magnification, 200×).

reflect the characteristic pathology of bacterial pneumonia [10, 25, 27].

Surprising aspects of 1918–1919 influenza-associated pneumonia fatalities included the following: (1) the high incidence of secondary pneumonia associated with standard bacterial pneumopathogens; (2) the frequency of pneumonia caused by both mixed pneumopathogens (particularly pneumococci and streptococci) and by other mixed upper respiratory–tract bacteria; (3) the aggressiveness of bacterial invasion of the lung, often resulting in "phenomenal" [30] numbers of bacteria and polymorphonuclear neutrophils, as well as extensive necrosis, vasculitis, and hemorrhage [20, 32, 33]; and (4) the predominance of bronchopneumonia and lobular pneumonia, as opposed to lobar pneumonia, consistent with diffuse predisposing bronchiolar damage [27–33].

Contemporary views of the natural history of severe influenza during the 1918–1919 influenza pandemic. By examining influenza autopsy materials from a range of patients in different stages of disease, pathologists in 1918–1919 identified the primary lesion in early severe influenza-associated pneumonia as desquamative tracheobronchitis and bronchiolitis extending diffusely over all or much of the pulmonary tree to the alveolar ducts and alveoli, associated with sloughing of bronchiolar epithelial cells to the basal layer, hyaline membrane formation in alveolar ducts and alveoli, and ductal dilation [20, 24, 27, 29–33].

Primary "panbronchitis" [35] was thought to reflect rapidly spreading epithelial cytolytic infection of the entire bronchial tree [32, 35, 36]; this was thought to have led to the secondary spread of enormous numbers of bacteria along the denuded bronchial epithelium to every part of the bronchial tree, following which focal bronchiolar infections broke through into the lung parenchyma. Secondary bacterial invasion and zones of vasculitis, capillary thrombosis, and necrosis surrounding areas of bronchiolar damage were seen in severe cases. As was true for the 58 autopsy cases we reviewed (see above), published autopsies for victims of the 1918–1919 pandemic generally showed histopathological asynchrony [20]. Repair, represented by early epithelial regeneration, capillary repair, and occasionally by fibrosis, was commonly seen in tissues sections from even the most fulminant fatal cases [20, 27, 32]. Among the ⩾60% of

Table 1. Bacterial culture results in autopsy series involving 96 postmortem cultures of lung tissue from victims of the 1918–1919 influenza pandemic.

Type of autopsy series	No. of results	No. (%) of cultures from which organism was recovered, by organism							
		Streptococcus pneumoniae	*Streptococcus hemolyticus*	*Staphylococcus aureus*	*Diplococcus intracellulare meningitidis*	Mixed pneumopathogens	*Bacillus influenzae*	Other bacteria	No growth
All military (n = 60)	3515	**855 (24.3)**	615 (17.5)	263 (7.5)	40 (1.1)	707 (20.1)	387 (11.0)	484 (13.8)	164 (4.7)
All civilian (n = 36)	1751	380 (21.7)	281 (16.0)	164 (9.4)	1 (<0.1)	**398 (22.7)**	132 (7.5)	339 (19.4)	56 (3.2)
All military and civilian (n = 96)	5266	**1235 (23.5)**	896 (17.0)	427 (8.1)	41 (0.8)	1105 (21.0)	519 (9.9)	823 (15.6)	220 (4.2)
All higher- quality military and civilian[a] (n = 68)	3074	712 (23.2)	553 (18.0)	238 (7.7)	21 (0.7)	**828 (26.9)**	144 (4.7)	353 (11.5)	225 (7.3)
Predominance of pneumopathogens not confirmed (n = 14)	1115	209 (18.7)	132 (11.8)	52 (4.7)	0 (0.0)	24 (2.2)	210 (18.8)	**402 (36.1)**	86 (7.7)

NOTE. The bacteria are listed by their common names in 1918. *Streptococcus pneumoniae* was cultured and (sometimes) typed with antisera into types I, II, IIa, III, and IV; type IV was generally regarded as containing a number of "untypeable types." *Streptococcus hemolyticus* probably corresponds to *Streptococcus pyogenes* in most cases; most observers distinguished *Staphylococcus aureus* from *Staphylococcus albus*, but in some cases observers noted only "*Staphylococcus*," which we categorized as "*aureus*" if the context suggested a pathogenic organism. *Diplococcus intracellulare meningitidis* corresponds to *Neisseria meningitidis*. *Bacillus influenzae* corresponds to *Haemophilus influenzae*. See Results for details about the "mixed pneumopathogens" and "other bacteria" categories. Many "other" organisms were undoubtedly untyped pneumococci and streptococci. Bold type indicates greatest percentage.

[a] A higher quality series was defined as a series in which lung tissue culture results reported, for all autopsies, both the presence and absence of negative culture results and the bacterial components of mixed culture results.

individuals who survived such severe pneumonia, severe chronic pulmonary damage was apparently uncommon [37, 38].

Bacteriologic studies in autopsy series during the 1918–1919 influenza pandemic. Negative lung culture results were uncommon in the 96 identified military and civilian autopsy series, which examined 5266 subjects (4.2% of results overall) (table 1; full bibliographic list available at http://www3.niaid.nih.gov/topics/Flu/1918/bibliography.htm). In the 68 higher-quality autopsy series, in which the possibility of unreported negative cultures could be excluded, 92.7% of autopsy lung cultures were positive for ⩾1 bacterium (table 1). Of these 96 series, 82 reported pneumopathogens in ⩾50% of lungs examined, either alone or in mixed culture results that included other bacteria (table 1). Outbreaks of meningococcal pneumonia complicating influenza also were documented [39]. Despite higher military case-fatality rates, the differences in the frequency with which specific bacteria were isolated from lung tissue cultures (table 1) and from culture of blood and pleural or empyema fluids (data not shown) were minimal. Many of the series were methodologically rigorous: in one study of approximately 9000 subjects who were followed from clinical presentation with influenza to resolution or autopsy [40], researchers obtained, with sterile technique, cultures of either pneumococci or streptococci from 164 of 167 lung tissue samples. There were 89 pure cultures of pneumococci; 19 cultures from which only streptococci were recovered; 34 that yielded mixtures of pneumococci and/or streptococci; 22 that yielded a mixture of pneumococci, streptococci, and other organisms (prominently pneumococci and nonhemolytic streptococci); and 3 that yielded nonhemolytic streptococci alone. There were no negative lung culture results.

In the 14 of 96 autopsy series that did not report the predominance of lung pneumopathogens [29, 36, 41–53], pneumopathogens accounted collectively for 37.4% of pneumonia deaths. The rest of the deaths were associated collectively with either culture of nonpneumopathogenic "other bacteria," such as nonhemolytic and viridans streptococci, "green-producing streptococci" [54], probably largely corresponding to α-hemolytic streptococci, uncharacterized diplostreptococci, *Micrococcus (Moraxella) catarrhalis, Bacillus (Escherichia) coli, Klebsiella* species, and complex mixed bacteria (36.1% of cultures). Cultures also yielded *Bacillus influenzae* (18.8%) and no bacterial growth (7.7%). These findings reflect rates of bacterial isolation similar to those of the series that reported the predominance of pneumopathogens (above and table 1), but with higher isolation rates for "other bacteria" offsetting the lower isolation rates for pneumococci, streptococci and staphylococci. It is noteworthy that pneumococcal typing antisera were unavailable in 11 of these 14 studies, and that many of the cultured "other" bacteria were reported as "gram-positive diplococci," "streptococci," or "diplostreptococci" (data not shown), consistent with the possibility that in this early era of bacterial typing, some of the unidentified organisms in the culture may have been pneumopathogens.

The predominant coinfecting microorganism in lung tissue cultures containing ⩾1 pneumopathogen was *Bacillus influenzae* (largely corresponding to the modern *Hemophilus influenzae*), an upper respiratory–tract organism not commonly found in pure culture of samples from any anatomical compartment [20, 36, 55]. *Bacillus influenzae* tended to appear early in symp-

Table 2. Bacterial culture results in autopsy series involving culture of blood and pleural fluid or empyema fluid from victims of the 1918–1919 influenza epidemic.

Type of autopsy series	No. of results	No. (%) of cultures from which organism was recovered, by organism							
		Streptococcus pneumoniae	*Streptococcus hemolyticus*	*Staphylococcus aureus*	*Diplococcus intracellulare meningitidis*	Mixed pneumopathogens	*Bacillus influenzae*	Other bacteria	No growth
Blood culture (n = 42)									
All military and civilian	1887	509 (27.0)	377 (20.0)	68 (3.6)	5 (0.3)	28 (1.5)	61 (3.2)	278 (14.7)	**561 (29.7)**
Pleural fluid or empyema fluid culture (n = 35)									
All military and civilian	1245	263 (21.1)	**539 (43.3)**	59 (4.7)	0 (0.0)	74 (5.9)	21 (1.7)	45 (3.6)	244 (19.6)

NOTE. The bacteria are listed by their common names in 1918. *Streptococcus pneumoniae* was cultured and (sometimes) typed with antisera into types I, II, IIa, III, and IV; type IV was generally regarded as containing a number of "untypeable types." *Streptococcus hemolyticus* probably corresponds to *Streptococcus pyogenes* in most cases; most observers distinguished *Staphylococcus aureus* from *Staphylococcus albus*, but in some cases observers noted only "*Staphylococcus*," which we categorized as "*aureus*" if the context suggested a pathogenic organism. *Diplococcus intracellulare meningitidis* corresponds to *Neisseria meningitidis*. *Bacillus influenzae* corresponds to *Haemophilus influenzae*. See Results for details about the "mixed pneumopathogens" and "other bacteria" categories. Many "other" organisms were undoubtedly untyped pneumococci and streptococci. Bold type indicates greatest percentage.

tomatic influenza in association with diffuse bronchitis and/or bronchiolitis, sometimes infiltrating the bronchiolar submucosa [35]; it caused seroconversion [56] and was then typically replaced by other secondary organisms.

Cultures of blood samples in 30 military and 12 civilian series, which examined a total of 1887 subjects (table 2), had positive results in 70.3% of cases and typically contained either pneumococci or streptococci in pure culture. Cultures of pleural or empyema fluid, reported in 23 military and 12 civilian series examining a total of 1245 subjects (table 2), revealed either streptococci or pneumococci as the most commonly recovered organism in all but 7 series: in 4 series mixed pneumopathogens predominated, and in 3 series *Staphylococcus aureus* predominated. Most subjects with positive culture results in the blood and pleural or empyema fluid series also had ⩾1 pneumopathogen cultured in samples from the lungs (data not shown).

Of 2007 pneumococcal isolates, 874 (43.5%) were serotyped by agglutination. Type I was isolated from 124 (14.2%) of 874 subjects; type II from 163 (18.6%); type IIa from 26 (3.0%); type III from 184 (21.1%); and type IV, a category containing diverse and, at the time, untypeable organisms, from 377 (43.1%).

Pathologic and bacteriologic information obtained from later pandemic and seasonal influenza cases. The viruses that caused the 1957 and 1968 pandemics were descendants of the 1918 virus in which 3 (the 1957 virus) or 2 (the 1968 virus) new avian gene segments had been acquired by reassortment [21]. Although lower pathogenicity resulted in far fewer deaths, hence fewer autopsies, most 1957–1958 deaths were attributable to secondary bacterial pneumonia, as had been the case in 1918. *Staphylococcus aureus*, a relatively minor cause of the 1918 fatalities, was predominant in the culture results from 1957–1958 [21, 57–61], and negative lung tissue cultures were more common, possibly as a result of the widespread administration of antibiotics [57, 58, 61]. The few relevant data from the 1968–1969 pandemic (see below) are consistent with information from the earlier 20th-century pandemics.

Human tracheobronchial biopsy studies performed since the 1957-1958 epidemic characterized the natural history of influenza virus infection as featuring rapid (within 24 h) development of bronchial epithelial necrosis, preservation of the basal layer, limited inflammatory response, and evidence of prompt repair [62], consistent with the observations of pathologists in 1918–1919.

DISCUSSION

In the most recent influenza pandemic that did not involve the use of antibiotics to suppress bacteria (the 1918–1919 pandemic), histological and bacteriologic evidence suggests that the vast majority of influenza deaths resulted from secondary bacterial pneumonia. Compelling evidence for this conclusion includes the examination of 58 recut and restained autopsy specimens that showed changes fully consistent with classical descriptions of extensive bacterial pneumonia [25], culture results from numerous international autopsy series, and consistent epidemiologic and clinical findings (table 3).

Between 1890 and 1950, most observers believed fatal influenza to be a polymicrobial infection in which an inciting agent of low pathogenicity (either a bacterium such as *Bacillus influenzae* or a "filter passing agent"—most of which have now been identified as viruses) acted synergistically with known pneumopathogenic bacteria [13, 14, 20, 33, 64–66]. This view was dramatically supported in 1917–1918 by the measles epidemics in US Army training camps, in which most deaths resulted from streptococcal pneumonia or, less commonly, pneumococcal pneumonia [20, 30, 32]. The pneumonia deaths during the in-

Table 3. Summary of evidence from the 1918–1919 influenza pandemic consistent with the conclusion that bacterial pneumonia, rather than primary viral pneumonia, was the cause of most deaths.

Evidence, by type	Relevant reference(s)
Pathologic Evidence	
Most autopsies revealed severe bacterial pneumonia caused by common upper respiratory organisms	[20, 27–33]
In type, pattern, and case-fatality rate, influenza-associated bacterial pneumonia, including chronic lobar pneumonia, was typical of pneumonia during periods when influenza was not prevalent; bronchopneumonia, associated with diffuse "panbronchitis," predominated	[25, 28, 33, 34]
At autopsy, early and/or extensive repair of what are now thought to be primary viral changes was evident; severe sequelae in pneumonia survivors were minimal	[20, 30, 32]
Pathologic picture of bacterial bronchopneumonia associated with influenza in 1918–1919 was strongly similar to the more highly fatal measles–bacterial bronchopneumonia epidemics of 1917–1918	[20, 27, 63]
Mixed pneumopathogen–associated pneumonia was more fatal than single-pneumopathogen pneumonia	[29]
Pneumonia cases exhibited uniformly diffuse and extensive tracheobronchitis and/or bronchiolitis, the severity of which correlated with pneumonia severity in degree and anatomical location	[29]
Demographic and/or epidemiologic evidence	
Most influenza cases were typical of cases seen today: mild, uncomplicated, and associated with full recovery	[13–17]
Mortality at all ages was associated with bacterial pneumonia rates, not with influenza attack rates or pneumonia case-fatality rates	[19, 21]
Children 5–15 years old in 1918–1919 had the highest attack rates but the lowest mortality rates, similar to low rates seen in 1889–1893 and immediately before and after the 1918–1919 pandemic—rates seemingly inconsistent with viral virulence alone	[14, 21]
Influenza-associated pneumonia incidence rates and influenza death rates were significantly higher in US military camps, which experienced bacterial "colonization epidemics"	[63]
Average time from influenza onset to pneumonia onset in ultimately fatal cases (~10 days) may be more consistent with bacterial than viral pneumonia	[29]
Treatment response evidence	
The near universal observation that strict bed rest early in the course of uncomplicated influenza prevented pneumonia and death is consistent with an effect of isolation from carriers of bacterial pathogens	[13, 14]

fluenza pandemic in 1918 proved so highly similar, pathologically, to the then-recent pneumonia deaths from the measles epidemics that noted experts considered them to be the result of one newly emerging disease: epidemic bacterial pneumonia precipitated by prevalent respiratory tract agents [20, 33, 63].

The question of whether the pathogenesis of severe influenza-associated pneumonia was primarily viral (i.e., assumed to be an unknown etiologic agent in 1918) or a combination of viral and bacterial agents was carefully considered by pathologists in 1918–1919, without definitive resolution [26, 33]. The issue was addressed anew in the early 1930s when Shope published a series of experimental studies that involved the just-discovered swine influenza A virus: severe disease in an animal model resulted only when the virus and *Hemophilus influenzae suis* were administered together [67]. In 1935, Brightman studied combined human influenza and streptococcal infection in a ferret intranasal inoculation model. Even though neither agent was pathogenic when administered alone, they were highly fatal in combination [68]. In rhesus monkeys, human influenza viruses given intranasally were not pathogenic, but could be made so by nasopharyngeal instillation of otherwise nonpathogenic bacteria [69]. During the 1940s, additional studies in ferrets, mice, and rats established that the influenza virus in combination with any of several pneumopathic bacteria acted synergistically to produce either a higher incidence of disease, a higher death rate, or a shortened time to death [70–73]; these effects could be mitigated or eliminated if antibiotics were given shortly after establishment of combined infection [73]. More recent data suggest that influenza vaccination may prevent bacterial disease [74].

As reviewed recently by McCullers [75], a body of experimental research during the last 3 decades has identified possible mechanisms by which coinfection with the influenza virus and bacteria might affect pathogenicity. These include viral neuraminidase (NA)–induced exposure of bacterial adherence receptors; bacterial NA-induced upregulation of influenza infection; interleukin 10–induced susceptibility to pneumococci and possibly staphylococci [76]; interferon type 1 effects [77]; viral PB1-F2 effects, the proaptotic and mitochondriopathic effects of which are correlated with enhanced bacterial infection [78]; and virus-induced desensitization to bacterial Toll-like receptor ligands [79].

We believe that the weight of 90 years of evidence (table 3), including the exceptional but largely forgotten work of an earlier generation of pathologists, indicates that the vast majority of pulmonary deaths from pandemic influenza viruses have resulted from poorly understood interactions between the infecting virus and secondary infections due to bacteria that colonize the upper respiratory tract. The data are consistent with a natural

history in which the virus, highly cytopathic to bronchial and bronchiolar epithelial cells, extends rapidly and diffusely down the respiratory tree, damages the epithelium sufficiently to break down the mucociliary barrier to bacterial spread, and if able to gain access to the distal respiratory tree—perhaps on the basis of receptor affinity [80]—creates both a direct pathway for secondary bacterial spread and an environment (cell necrosis and proteinaceous edema fluid) favorable to bacterial growth. It remains unresolved whether cocolonizing, nonpneumopathic upper respiratory–tract organisms such as *Bacillus (Hemophilus) influenzae* play an ancillary role, or are merely innocent bystanders. It is uncertain why *Hemophilus influenzae* was much less prominent in 1957–1958 and thereafter, but this phenomenon may relate to antibiotic use and conceivably, in recent years, to *Hemophilus influenzae b* vaccination of children.

The extraordinary severity of the 1918 pandemic remains unexplained. That the causes of death included so many different bacteria, alone or in complex combinations, argues against specific virulent bacterial clones. The pathologic and bacteriologic data appear consistent with copathogenic properties of the virus itself, perhaps related to viral growth, facility of cell-to-cell spread, cell tropism, or interference with or induction of immune responses. Certain observers believed that cotransmission of the influenza agent and of pneumopathogenic bacteria was responsible for many severe and fatal cases, especially during the October–November 1918 peak of mortality and case-fatality rates [81]. We speculate that any influenza virus with an enhanced capacity to spread to and damage bronchial and/or bronchiolar epithelial cells, even in the presence of an intact rapid reparative response, could precipitate the appearance of severe and potentially fatal bacterial pneumonia due to prevalent upper respiratory–tract bacteria.

In the modern era, the widespread use of antibiotics and the establishment of life-prolonging intensive care unit treatment make it more difficult than it was in 1918 to document the importance of bacterial lung infection for influenza-related mortality. Influenza-associated pneumonia patterns may now be influenced by the administration of pneumococcus, *Hemophilus influenzae b*, and meningococcus vaccine, and cases have tended to occur in elderly individuals, who rarely undergo autopsy. The 1968 influenza pandemic was mild, and autopsy studies were uncommon [21]. Fatal cases of influenza-associated viral pneumonia that are considered to be "primary" (i.e., with little or no bacterial growth) continue to be identified [82, 83]; however, their incidence appears to be low, even in pandemic peaks. The issue of the pathogenesis of fatal influenza-associated pneumonia remains important; the fact that even severe, virus-induced tissue damage is normally followed by rapid and extensive repair [20, 26] suggests that early and aggressive treatment, including antibiotics and intensive care, could save most patients [84, 85] and also underscores the importance of prevention and prophylaxis.

The 1918 pandemic and subsequent pandemics differed with respect to the spectrum and extent of secondary bacterial pneumonia (e.g., the switch in prevalence during the antibiotic era to predominantly staphylococcal secondary pneumonia, as opposed to streptococcal, pneumococcal, and mixed secondary pneumonia; and the greatly decreased involvement of *Bacillus [Hemophilus] influenzae*), suggesting that additional factors affect the level of influenza morbidity and mortality. These might include the use of antibiotics and antiviral agents, the rate of influenza vaccination and bacterial vaccination, and demographic and social factors. The aging population in the United States, the increasing number of persons living in nursing home facilities, and the number of persons who are immunosuppressed or affected by cardiac disease, renal disease, and/or diabetes mellitus all represent potential factors that might change the profile of morbidity and mortality during a future pandemic. For example, elderly persons in nursing homes are at risk for pneumonia caused by enteric organisms and sometimes by drug-resistant nosocomial organisms. The spread of bacteria such as methicillin-resistant *Staphylococcus aureus* and highly pathogenic clones of *Streptococcus pyogenes* pose more general risks [86].

The viral etiology of and timing of the next influenza pandemic cannot be predicted [87]. If, as some fear, a future pandemic is caused by a derivative of the current highly pathogenic avian H5N1 virus, lessons from previous pandemics may not be strictly applicable. Although histopathologic information concerning current human H5N1 infections is sparse [10], its pathogenic mechanisms may be atypical because the virus is poorly adapted to humans [88] and because, in certain experimental animal models [e.g., 89], some strains have induced severe pathology that differs from the findings associated with circulating human influenza viruses (which, in these models, cause disease resembling self-limited seasonal influenza in humans [90]). However, if an H5N1 virus were to fully adapt to humans, the clinicopathologic spectrum of associated disease could become more like that of previous pandemics.

If the next pandemic is caused by a human-adapted virus similar to those recognized since 1918, we believe the infection is likely to behave as it has in past pandemics, precipitating severe disease associated with prevalent colonizing bacteria. Recent reviews have discussed the importance of new and improved influenza antiviral drugs and influenza vaccines in controlling a pandemic [84, 91, 92]. The present work leads us to conclude that in addition to these critical efforts, prevention, diagnosis, prophylaxis, and treatment of bacterial pneumonia, as well as the stockpiling of antibiotics and bacterial vaccines [84, 85, 93], should be among the highest priorities in pandemic planning. We are encouraged that such considerations are already being discussed and implemented by the agencies and individuals responsible for such plans [94, 95].

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Immunization with SARS Coronavirus Vaccines Leads to Pulmonary Immunopathology on Challenge with the SARS Virus

Chien-Te Tseng[1,2], Elena Sbrana[1], Naoko Iwata-Yoshikawa[1,2], Patrick C. Newman[1], Tania Garron[1], Robert L. Atmar[3,4], Clarence J. Peters[1,2], Robert B. Couch[3,4]*

1 Department of Microbiology and Immunology, The University of Texas Medical Branch, Galveston, Texas, United States of America, 2 Center for Biodefense and Emerging Disease, The University of Texas Medical Branch, Galveston, Texas, United States of America, 3 Department of Medicine, Baylor College of Medicine, Houston, Texas, United States of America, 4 Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas, United States of America

Abstract

Background: Severe acute respiratory syndrome (SARS) emerged in China in 2002 and spread to other countries before brought under control. Because of a concern for reemergence or a deliberate release of the SARS coronavirus, vaccine development was initiated. Evaluations of an inactivated whole virus vaccine in ferrets and nonhuman primates and a virus-like-particle vaccine in mice induced protection against infection but challenged animals exhibited an immunopathologic-type lung disease.

Design: Four candidate vaccines for humans with or without alum adjuvant were evaluated in a mouse model of SARS, a VLP vaccine, the vaccine given to ferrets and NHP, another whole virus vaccine and an rDNA-produced S protein. Balb/c or C57BL/6 mice were vaccinated IM on day 0 and 28 and sacrificed for serum antibody measurements or challenged with live virus on day 56. On day 58, challenged mice were sacrificed and lungs obtained for virus and histopathology.

Results: All vaccines induced serum neutralizing antibody with increasing dosages and/or alum significantly increasing responses. Significant reductions of SARS-CoV two days after challenge was seen for all vaccines and prior live SARS-CoV. All mice exhibited histopathologic changes in lungs two days after challenge including all animals vaccinated (Balb/C and C57BL/6) or given live virus, influenza vaccine, or PBS suggesting infection occurred in all. Histopathology seen in animals given one of the SARS-CoV vaccines was uniformly a Th2-type immunopathology with prominent eosinophil infiltration, confirmed with special eosinophil stains. The pathologic changes seen in all control groups lacked the eosinophil prominence.

Conclusions: These SARS-CoV vaccines all induced antibody and protection against infection with SARS-CoV. However, challenge of mice given any of the vaccines led to occurrence of Th2-type immunopathology suggesting hypersensitivity to SARS-CoV components was induced. Caution in proceeding to application of a SARS-CoV vaccine in humans is indicated.

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* E-mail: rcouch@bcm.edu

Introduction

Severe acute respiratory syndrome (SARS) emerged in Guangdong, People's Republic of China, in late 2002, and spread to other countries in Asia and to Canada in the ensuing months [1–3]. Infection control efforts brought the infection under control by mid-2003 [4]. More than 8000 cases, including almost 800 deaths, were reported during the outbreak period [4]. Increasing age and comorbidity were risk factors for severe disease and death [5,6,7]. Since 2003, only sporadic cases have been reported; however, the possibility that SARS outbreaks could reemerge naturally or be deliberately released is a public health concern.

SARS is caused by a Coronavirus (SARS-CoV) [8,9]. Limited data are available about the ecology of SARS-CoV, but bats are thought to be the animal reservoir for the virus which may be transmitted to small mammals with exposure to these small animals as the source of human infections [10]. The clinical disease is similar to other severe acute respiratory infections, including influenza; the SARS case definition includes clinical, epidemiologic, and laboratory criteria [11,12]. A number of therapeutic efforts were employed for the disease in Asia and in Canada; however, no treatment of clear value was identified. Animal models were developed using mice, hamsters, ferrets and

nonhuman primates, and efforts to identify useful treatments and effective vaccines are ongoing.

Vaccine candidates for preventing SARS have been developed by various groups and include inactivated whole virus, spike (S) protein preparations, virus-like particles (VLPs), plasmid DNA and a number of vectors containing genes for SARS-CoV proteins [13–28]. Phase I studies in humans have been conducted with a whole virus vaccine and a DNA vaccine [29–30].

An early concern for application of a SARS-CoV vaccine was the experience with other coronavirus infections which induced enhanced disease and immunopathology in animals when challenged with infectious virus [31], a concern reinforced by the report that animals given an alum adjuvanted SARS vaccine and subsequently challenged with SARS-CoV exhibited an immunopathologic lung reaction reminiscent of that described for respiratory syncytial virus (RSV) in infants and in animal models given RSV vaccine and challenged naturally (infants) or artificially (animals) with RSV [32,33]. We and others described a similar immunopathologic reaction in mice vaccinated with a SARS-CoV vaccine and subsequently challenged with SARS-CoV [18,20,21,28]. It has been proposed that the nucleocapsid protein of SARS-CoV is the antigen to which the immunopathologic reaction is directed [18,21]. Thus, concern for proceeding to humans with candidate SARS-CoV vaccines emerged from these various observations.

The studies reported here were conducted to evaluate the safety, immunogenicity, and efficacy of different SARS-CoV vaccines in a murine model of SARS.

Materials and Methods

Tissue Cultures and Virus

Vero E6 tissue cultures [obtained from The American Type Culture Collection (ATCC), CRL:1586] were grown in Dulbecco's modified minimum essential medium (DMEM) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), 0.2% sodium bicarbonate and 10% fetal bovine serum (FBS). The Urbani strain of SARS-CoV was obtained from T.G. Ksiazek at the Centers for Disease Control and Prevention (Atlanta, GA), and a working stock of this virus was prepared by serially passaging a portion of the seed virus three times (p3) in Vero E6 cultures. The culture fluid from infected cells was clarified by low-speed centrifugation, filtered through a 0.45 µm filter, aliquoted, and stored at −80°C.

Vaccines

Four different SARS-CoV vaccines were evaluated in these studies (Table 1). Two whole virus vaccines were evaluated; one was prepared in Vero tissue cultures, zonal centrifuged for purification, and double-inactivated with formalin and UV irradiation, the DI vaccine (DIV); it was tested with and without alum adjuvant [16]. The other whole virus vaccine was prepared in Vero cells, concentrated, purified, inactivated with beta propiolactone and packaged with alum adjuvant (BPV) [13]. A recombinant DNA spike (S) protein vaccine (SV) was produced in insect cells and purified by column chromatography was tested with and without alum adjuvant [17]. The fourth vaccine (the VLP vaccine) was a virus-like particle vaccine prepared by us as described previously; it contained the SARS-CoV spike protein (S) and the Nucleocapsid (N), envelope (E) and membrane (M) proteins from mouse hepatitis coronavirus (MHV) [20].

Animals

Six- to eight-week-old, female Balb/c and C57BL/6 mice (Charles River Laboratory, Wilmington, MA), were housed in cages covered with barrier filters in an approved biosafety level 3 animal facility maintained by the University of Texas Medical Branch (UTMB) at Galveston, Texas. All of the experiments were performed using experimental protocols approved by the Office of Research Project Protections, Institutional Animal Care and Use Committee (IACUC), University of Texas Medical Branch and followed National Institutes of Health and United States Department of Agriculture guidelines.

Study Design

Three different experiments, performed for comparing different vaccines, are reported here. Adjuvanted (alum) and non-adjuvanted (PBS) vaccines were obtained from the NIH/BEI resource. Groups of mice (N = 12–13 per group) were administered various dosages of each vaccine intramuscularly (IM) on days 0 and 28; mice given only PBS, alum, trivalent inactivated influenza vaccine or live SARS-CoV were included as controls in various experiments. On day 56, five mice from each group were sacrificed for assessing serum neutralizing antibody titers and lung histopathology; the remaining seven or eight mice in each group were challenged with 10^6TCID$_{50}$/60 µl of SARS-CoV intranasally (IN). Challenged mice were euthanized on day 58 for determining virus quantity and preparing lung tissue sections for histopathologic examination.

Neutralizing Antibody Assays

Mice were anesthetized with isoflurane and then bled from the retro-orbital sinus plexus. After heat inactivation at 56°C for 30 minutes, sera were stored at −80°C until tested. Assays for virus-specific neutralizing antibodies were performed on serial 2-fold diluted samples of each serum using 2% FBS-DMEM as the diluent in 96-well tissue culture plates (Falcon 3072); the final volume of the serially diluted samples in each well was 60 µl after addition of 120 TCID$_{50}$ of SARS-CoV in 60 µl into each well. The beginning dilution of serum was 1:20. The dilutions were incubated for 45–60 minutes at room temperature; then 100 µl of each mixture was transferred into duplicate wells of confluent Vero E6 cells in 96-well microtiter plates. After 72 hours of incubation, when the virus control wells exhibited advanced virus-induced CPE, the neutralizing capacity of individual serum samples were assessed by determining the presence or absence of cytopathic effect (CPE). Neutralizing antibody titers were expressed as the reciprocal of the last dilution of serum that completely inhibited virus-induced CPE.

Collection and Processing of Lungs for Histology and Virus Quantity

Two days post SARS-CoV challenge, mice were euthanized and their lungs were removed. Lung lobes were placed in 10% neutral buffered formalin for histological examination and immunohistochemistry (IHC), as described previously [34,35]. For virus quantitation, the remaining tissue specimen was weighed and frozen to −80°C. Thawed lung was homogenized in PBS/10% FBS solution using the TissueLyser (Qiagen; Retsch, Haan, Germany). The homogenates were centrifuged and SARS-CoV titers in the clarified fluids were determined by serial dilution in quadruplicate wells of Vero E6 cells in 96-well plates. Titers of virus in lung homogenates were expressed as TCID$_{50}$/g of lung ($\log_{10}$); the minimal detectable level of virus was 1.6 to 2.6 $\log_{10}$ TCID$_{50}$ as determined by lung size.

Table 1. Experimental Groups for Evaluation of SARS Coronavirus Vaccines.

Group	Exp 1[1] Vaccine Comparisons	Exp 2[1] Higher SV Dosage plus DIV and BPV Comparisons	Exp 3[1,3] Mouse and Vaccine Specificity
1	DIV/1 μg[2]	PBS	PBS-PBS
2	DIV/0.5 μg	Live virus	PBS
3	DIV/0.25 μg	SV/9 μg	Live virus
4	DIV/0.125 μg	SV/3 μg	Flu vaccine
5	DIV/1 μg + alum	SV/1 μg	DIV/1 μg
6	DIV/0.5 μg + alum	SV/9 μg + alum	DIV/1 μg + alum
7	DIV/0.25 μg + alum	SV/3 μg + alum	BPV/undil + alum
8	DIV/0.125 μg + alum	SV/1 μg + alum	PBS-PBS
9	SV/2 μg[2]	DIV/1 μg	PBS
10	SV/1 μg	DIV/0.25 μg (50 μl)	Live virus
11	SV/0.5 μg	DIV/1 μg + alum	Flu vaccine
12	SV/0.25 μg	DIV/0.25 μg + alum (50 μl)	DIV/1 μg
13	SV/2 μg + alum	BPV/undil + alum[2]	DIV/1 μg + alum
14	SV/1 μg + alum	BPV/undil + alum (25 μl)	BPV/undil + alum
15	SV/0.5 μg + alum		
16	SV/0.25 μg + alum		
17	VLP/2 μg[2]		
18	VLP/2 μg + alum		
19	Alum		
20	PBS		

[1]Design = All experiments in Balb/c mice except as noted in Exp 3. Each group contained 12–13 mice; all were given 100 μl of vaccine IM at dosages with or without alum as indicated on days 0 and 28 except as noted. Five mice in each group were sacrificed on day 56 for serum antibody; remaining mice were given 10^6 $TCID_{50}$ of SARS-CoV intranasal on day 56 and sacrificed on day 58 for virus and lung histology.
[2]DIV/dosage = Vaccine DIV = Zonal centrifuge purified doubly inactivated (formalin and UV) whole virus SV/dosage = Vaccine SV = Recombinant baculovirus expressed S glycoprotein of SARS-CoV VLP/dosage = Vaccine VLP = Virus-like particles containing SARS-CoV S glycoprotein and E, M, and N proteins from mouse hepatitis coronavirus BPV/dosage = Vaccine BPV = Purified beta propiolactone inactivated whole virus plus alum.
[3]Experiment 3 = Groups 1 to 7 were Balb/c mice; groups 8 to 14 were C57BL/6 mice. Flu vaccine was licensed trivalent 2009-10 formulation of high dosage vaccine (60 μg of HA of each strain). Groups 1 and 8 were given PBS (placebo) and challenged with PBS; all others were challenged with live SARS-CoV.
doi:10.1371/journal.pone.0035421.t001

Histopathology

Evaluations for histopathology were done by pathologists masked as to the vaccine/dosage of each specimen source; numeric scores were assigned to assess the extent of pathologic damage and the eosinophilic component of the inflammatory infiltrates.

Statistical Analysis

Neutralizing antibody titers, lung virus titers, histopathologic lesion score and eosinophilic infiltration scores were averaged for each group of mice. Comparisons were conducted using parametric and nonparametric statistics as indicated.

Results

Experiments

The three experiments performed, vaccines and dosages used and controls for each experiment are shown in Table 1. The vaccines were evaluated for immunogenicity and efficacy; however, because of the previous report of immunopathology on challenge of ferrets and nonhuman primates that had been vaccinated with a whole virus adjuvanted vaccine and mice that had been vaccinated with a VLP vaccine, the primary orientation was to assess for immunopathology among animals in relation to type of vaccine, dosage, serum antibody responses, and virus infection. The vaccine preparations were made for human trials so identifying a preparation that was likely to be both safe and protective in humans was desired. The rationale for each experiment is described.

Comparison of Vaccines (Experiment 1). To differentiate between vaccines, three vaccine preparations were simultaneously evaluated, the double-inactivated (formalin and UV) whole virus vaccine (DIV), the rDNA-expressed S protein vaccine (SV), and the previously evaluated chimeric viral-like particle vaccine (VLP) that had led to immunopathology with virus challenge [16,17,20].

Geometric mean serum neutralizing antibody titers for each group on day 56 are shown in figure 1A. Geometric mean titers for those given a nonadjuvanted or alum adjuvanted vaccine were not different for the double-inactivated whole virus vaccine (DIV), and the VLP vaccine, ($p>0.05$, student's t-test), but were different for the S protein vaccine (SV) ($p = 0.001$, student's t test). Geometric mean titers for the different dosage groups given the DI vaccine (DIV) with alum and those for the groups given the S protein vaccine (SV) with or without alum were significantly different ($p = 0.007$, $p = 0.028$, and $p = 0.01$, respectively, Kruskall-Wallis) while the geometric means for those dosage groups given the DI vaccine (DIV) without alum were not ($p>0.05$, Kruskall-Wallis). In a multiple regression analysis, postvaccination titers for the DI vaccine (DIV) were significantly increased by both alum and higher dosage (for alum, $p = 0.012$, for dosage, $p < 0.001$); for the S protein vaccine (SV), only alum increased responses ($p = 0.001$).

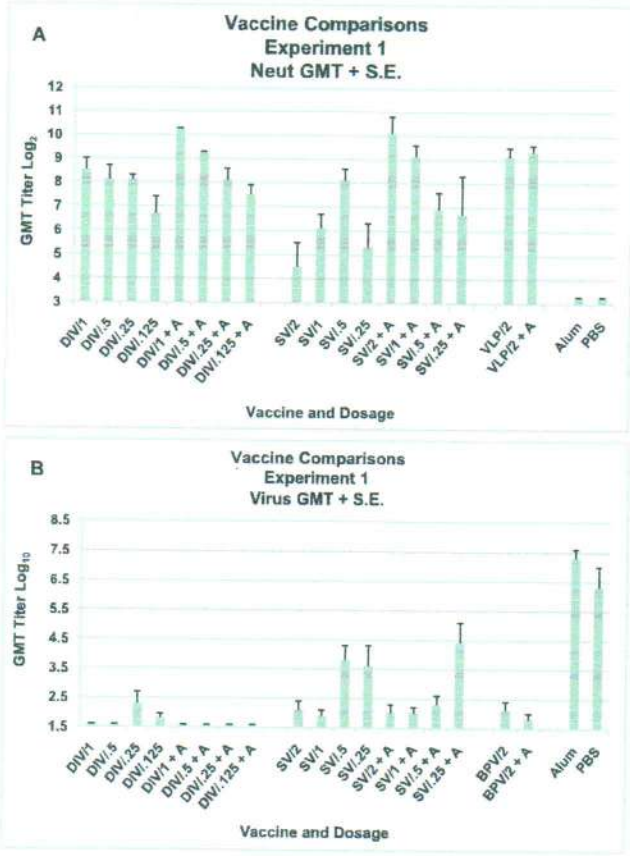

Figure 1. Vaccine Comparisons of Three SARS-CoV Vaccines, Experiment 1. Serum neutralizing (neut) antibody and lung virus titers for each vaccine dosage group. A. Geometric mean serum antibody titer as log_2 and standard error of the mean (S.E.) on day 56 for each vaccine dosage group. Seven to eight mice per group. Vaccines: double inactivated whole virus (DIV), recombinant S protein (SV), viral-like particle vaccine (VLP), with alum (+A). Five mice per group were given 0.1 ml of vaccine intramuscularly on days 0 and 28. B. Geometric mean virus titer (log_{10} $TCID_{50}$/g) and standard error of the mean (S.E.) in lungs on day 58 (two days after SARS-CoV challenge) for each vaccine dosage group. Analyses: A. GMT with compared to without alum: DIV p>.05, VLP p>.05, SV p = .001. GMT for different vaccine dosage: DIV with alum p = .007, DIV without alum p>.05, SV with alum p = .028, SV without alum p = .01. Multiple regression: GMT increased for alum p = .012 and dosage p<.001, for SV alum only p = .001. B. GMT for all DIV groups not different p>.05, GMT for SV group without alum p .008 and with alum p .023. GMT for VLP group is not different p>.05.
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Two days after challenge, lungs were obtained from all animals for virus quantitation and histology. CoV titers are shown in figure 1B. Geometric mean lung titers in the alum and PBS control groups were $10^{7.3}$ and $10^{6.3}$ $TCID_{50}$/g, respectively. All vaccine groups exhibited lower titers or no detectable virus on day two after challenge. None of the animals given any of the alum-adjuvanted DI vaccine (DIV) dosages and only an occasional animal in the lower dosages of nonadjuvanted vaccine yielded virus (Kruskall-Wallis and Mann Whitney U tests, p>0.05 for all comparisons). All groups given the S protein vaccine (SV) yielded virus after challenge and the differences between groups were significant (p = 0.002 for all groups, p = 0.023 for alum and p = 0.008 for no adjuvant, Kruskall-Wallis); also, geometric mean titers were higher for the groups given lower vaccine dosages. Geometric mean titers for the VLP vaccine groups were similar (p>0.05).

In the vaccine comparison experiment, lung lesion scores for histopathology were graded for individual animals on a scale of 0 to 4 where 0–2 represented degree of cellular infiltration and 3–4 represented the degree of bronchiolar epithelial cell necrosis and airway cellular debris (figure 2A). As shown, all animals exhibited pathologic changes after challenge including those animals with no measurable virus on day two suggesting virus infection had occurred but was not detectable on day two because of a short duration of infection or neutralization of virus by antibody in the lung during processing. The higher scores (>3) in some groups related primarily to the fact that virus infection had induced inflammatory infiltrates and epithelial cell necrosis with desquamation of the epithelium and collection of cellular debris in airways of these animals. Mean score differences were noted among the various vaccines (p = <0.001, Anova). Those groups given the DI vaccine (DIV) without alum had higher mean scores than did those given DI vaccine (DIV) with alum (p = 0.001, Mann-Whitney U); similarly, the group given the VLP vaccine without alum had a higher mean score than for those given VLP vaccine with alum (p = 0.008, Mann-Whitney U). Post hoc comparisons for the three different vaccines indicated that the DI vaccine (DIV) group overall had lower lesion scores than either the S protein vaccine (SV) group or the alum and PBS control groups (p = 0.001 comparing the DI and S protein vaccines (DIV and SV) and p<0.001 for DIV vs. control groups, Tukey HSD and Dunnett t, respectively), but not the VLP vaccine group (p>0.05, Tukey HSD). The S protein vaccine group (SV) was also lower overall than the control groups (p = 0.048, Dunnett t).

When the characteristics of the infiltrates were compared, animals given alum or PBS exhibited epithelial cell necrosis and peribronchiolar and perivascular mononuclear cell infiltrates consistent with epithelial cell infection and an inflammatory response seen in viral infections. In addition to mononuclear cells, however, infiltrates among vaccinated animals contained neutrophils and eosinophils that were not seen in the lesions of the animals that had been previously given PBS or alum only (figure 2B) suggesting a T helper cell type 2 hypersensitivity reaction; increased eosinophils are a marker for a Th2-type hypersensitivity reaction. Percent eosinophils was lower in these vaccinated animals (mean 1–3.2%) than had been seen in animals given VLP vaccines in the earlier study (mean 13.2±9.6% and 22±9.9% of cells for VLP with PBS or alum, respectively in that study) but no (0%) eosinophils were seen in the lung infiltrates of control animals in this experiment. This pattern of excess eosinophils in cellular infiltrates seen in lung sections from animals given vaccine and not in control animals was as seen in the earlier study with VLP vaccine and those later with other vaccines although the percent eosinophils was lower in this study.

The mean percent eosinophils differed between groups (p<0.001, Anova). Overall, the percent was lower for the groups given the DI and S protein alum adjuvanted vaccines than for the corresponding nonadjuvanted group (p = 0.049 for DIV and 0.001 for SV, Mann-Whitney U). For the vaccines, the eosinophil mean percentages were lower for the S protein vaccine (SV) than for either the DI vaccine (DIV) or VLP vaccine (DIV vs. SV, p = 0.002; VLP vs. SV, p = <0.001, Tukey HSD). Additionally, eosinophil percentages for all three vaccines, including the S protein vaccine, were significantly greater than the controls (SV, DIV and VLP vaccine, p<0.001 for each, Tukey HSD).

Higher Dosages of the S Protein Vaccine Plus the bp Inactivated Whole Virus Vaccine, Experiment 2. This experiment was conducted to verify the findings in the initial

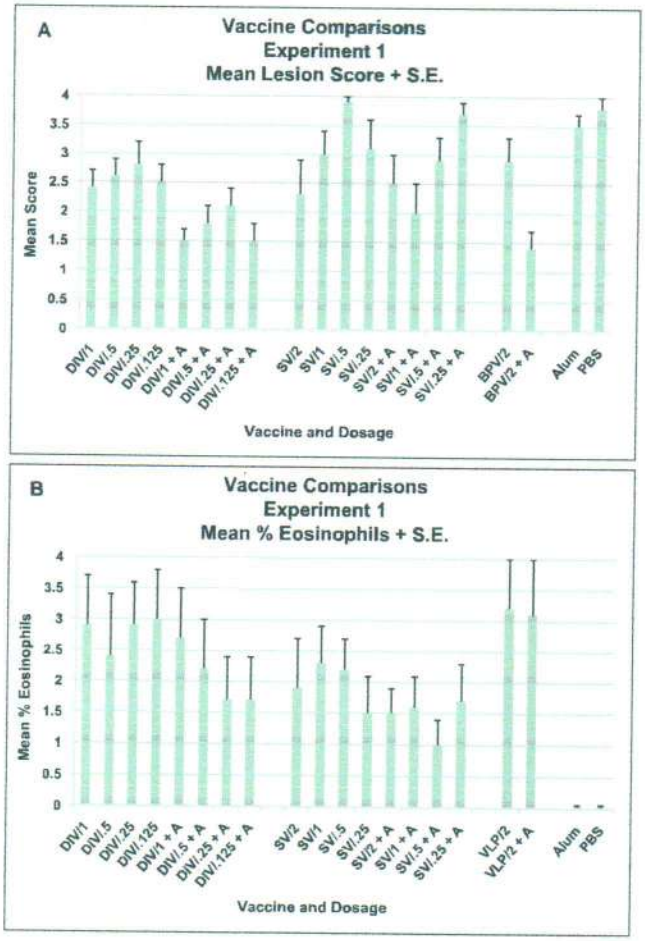

Figure 2. Vaccine Comparisons of Three SARS-CoV Vaccines, Experiment 1. Mean lung cellular infiltration/lesion pathology and percent eosinophils in infiltrates for each vaccine dosage group two days after challenge with SARS-CoV. A. Mean lesion score and standard error of the mean (S.E.) for each vaccine dosage group. All mice exhibited lung histopathology. Scores are mean of scores for seven to eight mice per group. Scoring. 0 – no pathology, 1 and 2 – (1) minimal (2) moderate peribronchiole and perivascular cellular infiltration, 3 and 4 – 1 and/or 2 plus minimal (3) or moderate (4) epithelial cell necrosis of bronchioles with cell debris in the lumen. B. Mean percent eosinophils on histologic evaluation for seven to eight mice in each vaccine dosage group. Mean for each mouse is the mean percent eosinophils on five separate microscopy fields of lung sections. Analyses: A. Mean lesion scores were different $p<.001$. DIV without alum greater than with alum $p=.001$, VLP without alum greater than with alum $p=.008$. Posthoc comparisons: DIV lower than SV $p=.001$ and controls $p<.001$ but not VLP $p>.05$. SV lower than controls p .048. B. Mean percent eosinophils were different $p<.001$. Mean percent eosinophils lower for DIV with alum than without alum $p=.049$ and lower for SV with alum than without alum $p=.001$. Mean percent eosinophils lower for SV than DIV $p=.002$ or VLP. $P=<.001$. Mean percent eosinophils greater than controls for DIV, SV and VLP, all three vaccines $p<.001$.
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experiment of a hypersensitivity immunopathologic-like reaction after SARS-CoV challenge of vaccinated animals, to determine if a higher dosage of the S protein vaccine (SV) would suppress infection and still exhibit a similar reaction, and whether the original β propiolactone inactivated whole virus vaccine (BPV) that had shown an immunopathologic-like reaction after challenge of vaccinated ferrets and nonhuman primates exhibited a similar immunopathologic reaction in the mouse model [13,14]. Additionally, a live virus "vaccination" group was added in this experiment for comparison of challenge results following vaccinations with inactivated vaccines to those following earlier infection.

Serum neutralizing antibody responses are shown in figure 3A. The bp inactivated vaccine (BPV), was only available at one dosage with alum so a smaller volume (25 μl) was given to one group for a dosage comparison. Geometric mean titers for the groups given the alum adjuvanted version of the DI and the S protein vaccines were greater than for the unadjuvanted vaccine (DIV $P=0.014$, SV $p<0.001$, student's t test). In multiple regression analysis, titers were also significantly increased after both the DI and S protein vaccines with use of alum ($p\leq0.01$); no dosage effect was noted. The geometric mean neutralizing antibody titers of the two bp inactivated vaccine groups (BPV) were different ($p=0.039$, Mann-Whitney U).

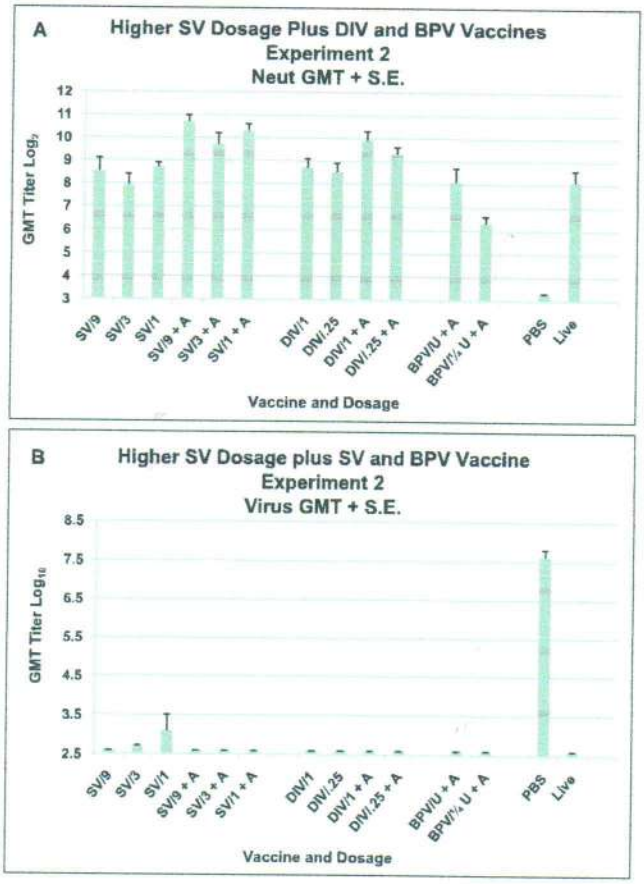

Figure 3. Higher Dosages of SV Vaccine plus DIV and BPV Vaccine Comparisons, Experiment 2. Serum neutralizing (neut) antibody and lung virus titers for each vaccine dosage group. A. Geometric mean serum antibody titer and standard error of the mean (S.E.) on day 56 for each vaccine dosage group. Five mice per group given 0.1 ml of vaccine intramuscularly on days 0 and 28. B. Geometric mean virus titer (log_{10} $TCID_{50}$/g) and standard error of the mean (S.E.) in lungs on day 58 (two days after SARS-CoV challenge) for each vaccine dosage group. Seven to eight mice per group. Vaccines: double inactivated whole virus (DIV), recombinant S protein (SV), β propiolactone inactivated whole virus (BPV) with alum (+A). Analyses: A. GMT with alum greater than without alum: SV $p<.001$, DIV $p=.014$. GMT for the two BPV groups are different $p=.039$. Multiple regression: DIV and SV increased with alum $p\leq.01$, no dosage effect $p>.05$.
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Two days after challenge with 10^6 $TCID_{50}$ of SARS-CoV, titers in mice given PBS varied between $10^{7.0}$ and $10^{8.0}$ $TCID_{50}$ per g of tissue; one vaccinated animal in the group given the S protein vaccine (SV) at the 3 μg and the 1 μg dosage without alum yielded virus but all other animals in all other groups were culture negative for virus (figure 3B).

Shown in figure 4A are the mean lesion scores on histologic evaluations. The scoring system for experiments two and three were developed by a replacement pathologist who preferred a scale of 0 to 3 which corresponded to a judgment of mild, moderate or severe (figure 4A). Mean lesion scores for this grading system overall were significantly different from each other ($p<0.001$, Anova) and scores were lower for the S protein vaccine than for either of the whole virus vaccines (SV versus DIV and BPV, $p<0.001$ and $p=0.006$, respectively, Tukey HSD). Of interest is that those given live virus and then challenged with live virus two months later exhibited an infiltrative disease severity comparable to the PBS and vaccinated groups despite no detectable virus on day two, again suggesting some degree of infection may have occurred earlier.

The mean eosinophil scores for the lung infiltrations were lower for the S protein vaccine groups [SV vs. DIV $p<0.001$; SV vs. BPV, $p<0.001$, Tukey HSD]; however, they were clearly greater than seen in those given PBS or live virus earlier ($p<0.001$, Tukey HSD) (figure 4B).

Representative photo micrographs of lung sections from mice in this experiment two days after challenge with SARS-CoV are shown in figure 5. The pathologic changes were extensive and similar in all challenged groups (H & E stains). Perivascular and peribronchial inflammatory infiltrates were observed in most fields along with desquamation of the bronchial epithelium, collections of edema fluid, sloughed epithelial cells, inflammatory cells and cellular debris in the bronchial lumen. Large macrophages and swollen epithelial cells were seen near lobar and segmental bronchi, small bronchioles and alveolar ducts. Necrotizing vasculitis was prominent in medium and large blood vessels, involving vascular endothelial cells as well as the tunica media, and included lymphocytes, neutrophils, and eosinophils in cellular collections. Occasional multinucleated giant cells were also seen. The eosinophil component of infiltrates was very prominent in animals vaccinated with the experimental vaccine preparations when compared to animals mock-vaccinated using PBS, or those exposed earlier to live virus (figure 6); few to no eosinophils were seen in those lung sections. Thus, while pathology was seen in sections from the control mice, the hypersensitivity-type pathologic reaction with eosinophils was not seen. The morphological identification of eosinophils in H&E stains was supported by using Giemsa stain to highlight intracytoplasmic granules in selected lung sections (not shown), and confirmed by immunostaining with antibodies against mouse eosinophil major basic protein (provided by the Lee Laboratory, Mayo Clinic, Arizona) [36].

The different groups of vaccinated animals showed similar trends in severity of pathology and of eosinophils in inflammatory infiltrates; however, the DIV and BPV preparations at high dosage tended to produce a greater infiltration with eosinophils.

Mouse and Vaccine Specificity (Experiment 3). Experiment 3 was performed to evaluate vaccine and mouse strain specificity. SARS-CoV vaccines used were the DI vaccine (DIV) with and without alum and the bp inactivated vaccine (BPV), which contains alum, at the highest dosage. For mouse strain specificity, Balb/c mice were included for consistency between experiments; C57BL/6 mice were given the same vaccines and dosages as Balb/c mice for comparison as C57BL/6 mice do not exhibit a bias for Th2 immunologic responses as do

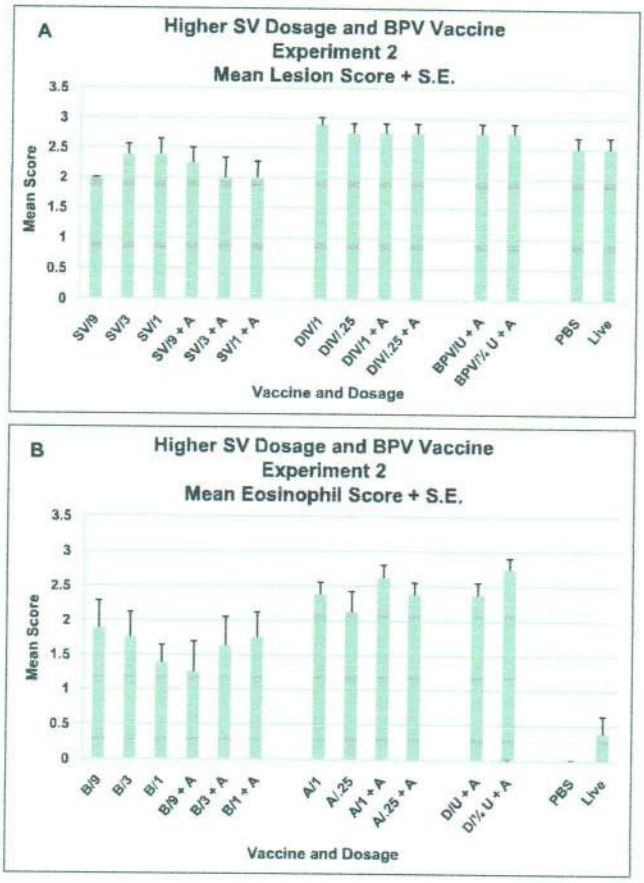

Figure 4. Higher Dosages of SV Vaccine plus DIV and BPV Vaccine Comparisons, Experiment 2. Mean lung cellular infiltration/lesion pathology and mean percent eosinophils in infiltrates for each vaccine dosage group two days after challenge with SARS-CoV. A. Mean lesion score and standard error of the mean (S.E.) for each vaccine dosage group. Scores are mean of scores for seven to eight mice per group. Scoring - 0 - no definite pathology, 1 - mild peribronchiole and perivascular cellular infiltration, 2 - moderate peribronchiole and perivascular cellular infiltration, 3 - severe peribronchiolar and perivascular cellular infiltration with thickening of alveolar walls, alveolar infiltration and bronchiole epithelial cell necrosis and debris in the lumen. Ten to 20 microscopy fields were scored for each mouse lung. B. Mean score and standard error of the mean (S.E.) for eosinophils as percent of infiltrating cells for each vaccine dosage group. Scores are mean of scores for seven to eight mice per group. Scoring: 0 - <5% of cells, 1 - 5–10% of cells, 2 - 10–20% of cells, 3 - >20% of cells. Ten to 20 microscopy fields were scored for each mouse lung. Analyses: A. Mean lesion scores were different $p<.001$. Mean scores were lower for SV than DIV $p<.001$ and less than BPV $p=.006$. B. Mean eosinophil scores were lower for SV than DIV $p<.001$ and less than BPV $p<.001$. Eosinophil scores greater for SV than PBS or live virus $p<.001$.
doi:10.1371/journal.pone.0035421.g004

Balb/c mice [37–39]. PBS and live virus controls were again included and trivalent 2010-11 formulation influenza vaccine at a dosage of 12 μg per component was given to assess vaccine specificity.

Neutralizing antibody titers are shown in figure 7A. Geometric mean titers for the highest dose of the DI vaccine were higher for those vaccine groups in the Balb/c mice than the C57BL/6 mice but only the nonadjuvanted DI vaccine group was significantly higher ($p=0.008$, Mann Whitney U). The serum antibody responses after BPV and live virus administration were similar for the two mouse strains. After challenge, mean lung virus titers

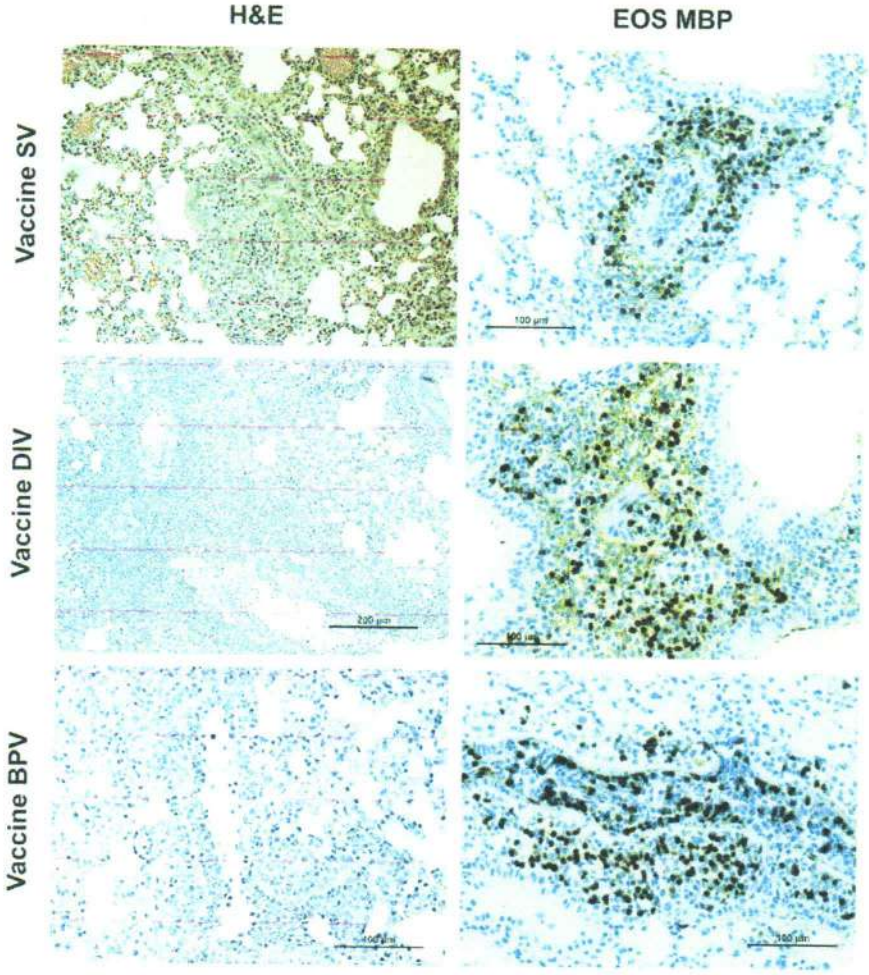

Figure 5. Photographs of Lung Tissue. Representative photomicrographs of lung tissue two days after challenge of Balb/c mice with SARS-CoV that had previously been given a SARS-CoV vaccine. Lung sections were separately stained with hematoxylin and eosin (H&E) and an immunohistochemical protocol using an eosinophil-specific staining procedure with a monoclonal antibody to a major basic protein of eosinophils. DAB chromogen provided the brown eosinophil identity stain. The procedure and antibody were kindly provided by the Lee Laboratory, Mayo Clinic, Arizona. The H&E stain column is on the left and eosinophil-specific major basic protein (EOS MBP) stain column is on the right. Vaccines: double inactivated whole virus (DIV), β propiolactone inactivated whole virus vaccine (BPV). As shown in the images, eosinophils are prominent (brown DAB staining) in all sections examined. Exposure to SARS-CoV is associated with prominent inflammatory infiltrates characterized by a predominant eosinophilic component.
doi:10.1371/journal.pone.0035421.g005

were similar for the PBS control challenged mice of both mouse strains ($10^{6.7-7.3}$ $TCID_{50}$/g lung) (figure 7B). None of the Balb/c mouse groups given either vaccine or live virus earlier yielded virus after challenge but some virus was detected in C57BL/6 mice given the DIV without alum and the BPV with alum (C57BL/6 versus Balb/c, $p = 0.004$, Mann Whitney U).

Mean lung lesion scores two days after challenge were similar for all groups and indicated a moderate to severe degree of cellular infiltration ($p>0.05$ for each, Anova) (figure 8A). However, eosinophil scores were significantly different between groups ($p<0.001$, Anova) with significantly lower scores for nonvaccine groups than for vaccine groups of both mouse strains ($p<0.001$ for all comparable group comparisons, Tukey's HSD). Eosinophil scores for the vaccine groups were not different between the two mouse strains ($p>0.05$, t test) (figure 8B). Photomicrographs of the different vaccine and mouse strain groups are shown in figure 9. Both vaccines in both mouse strains exhibited significant cellular infiltrations that included numerous eosinophils as shown in the MBP stained sections, a finding consistent with a hypersensitivity component of the pathology. Prior influenza vaccine did not lead to an eosinophil infiltration in the lung lesions after challenge.

Discussion

The emergence of the disease SARS and the rapid identification of its severity and high risk for death prompted a rapid mobilization for control at the major sites of occurrence and at the international level. Part of this response was for development of vaccines for potential use in control, a potential facilitated by the rapid identification of the causative agent, a new coronavirus [8–9]. Applying the principles of infection control brought the epidemic under control but a concern for reemergence naturally or a deliberate release supported continuation of a vaccine development effort so as to have the knowledge and capability necessary for preparing and using an effective vaccine should a need arise. For this purpose, the National Institute of Allergy and Infectious Diseases supported preparation of vaccines for evaluation for potential use in humans. This effort was hampered by the

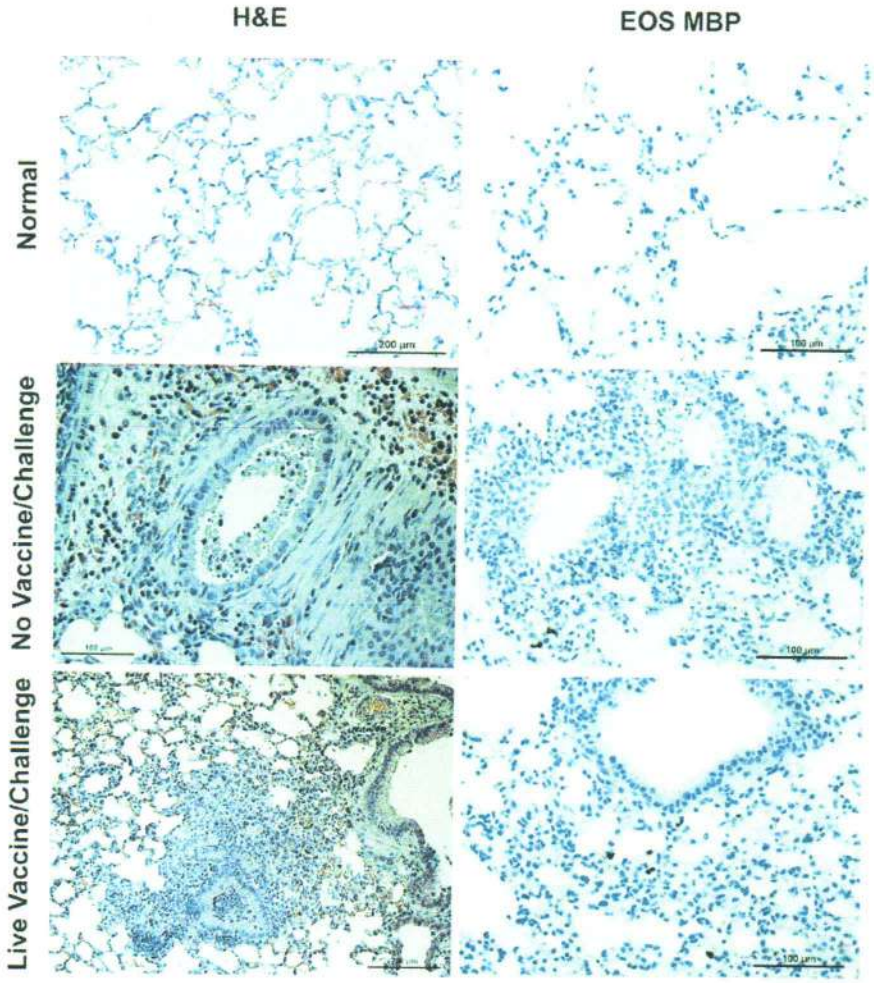

Figure 6. Photomicrographs of Lung Tissue. Representative photomicrographs of lung tissue from unvaccinated unchallenged mice (normal) and from Balb/c mice two days after challenge with SARS-CoV that had previously been given PBS only (no vaccine) or live virus. H&E and immunohistochemical stains for eosinophil major basic protein were performed as described for figure 5. The H&E column is on the left and the Eos MBP column is on the right. Shown are sections from normal mice (no vaccine or live virus) and mice given PBS (no vaccine) or live SARS-CoV and then challenged with SARS-CoV. As shown in the middle and bottom row images, although exposure to SARS-CoV elicits inflammatory infiltrates and accumulation of debris in the bronchial lumen, eosinophils in all groups remain within normal limits.
doi:10.1371/journal.pone.0035421.g006

occurrence in the initial preclinical trial of an immunopathogenic-type lung disease among ferrets and Cynomolgus monkeys given a whole virus vaccine adjuvanted with alum and challenged with infectious SARS-CoV [14]. That lung disease exhibited the characteristics of a Th2-type immunopathology with eosinophils in the lung sections suggesting hypersensitivity that was reminiscent of the descriptions of the Th2-type immunopathologic reaction in young children given an inactivated RSV vaccine and subsequently infected with naturally-occurring RSV [32–33]. Most of these children experienced severe disease with infection that led to a high frequency of hospitalizations; two children died from the infection [33,40,41]. The conclusion from that experience was clear; RSV lung disease was enhanced by the prior vaccination. Subsequent studies in animal models that are thought to mimic the human experience indicate RSV inactivated vaccine induces an increased $CD4^+$ T lymphocyte response, primarily of Th2 cells and the occurrence of immune complex depositions in lung tissues [32,42,43]. This type of tissue response is associated with an increase in type 2 cytokines including IL4, IL5, and IL13 and an influx of eosinophils into the infected lung; [32,33,42,44]. Histologic sections of tissues exhibiting this type of response have a notable eosinophilic component in the cellular infiltrates. Recent studies indicate that the Th2-type immune response has both innate and adaptive immune response components [33,43].

In addition to the RSV experience, concern for an inappropriate response among persons vaccinated with a SARS-CoV vaccine emanated from experiences with coronavirus infections and disease in animals that included enhanced disease among infected animals vaccinated earlier with a coronavirus vaccine [31]. Feline infectious peritonitis coronavirus (FIPV) is a well-known example of antibody-mediated enhanced uptake of virus in macrophages that disseminate and increase virus quantities that lead to enhanced disease [31,45]. Antigen-antibody complex formation with complement activation can also occur in that infection and some other coronavirus infections in animals. Thus, concern for safety of administering SARS-CoV vaccines to humans became an early concern in vaccine development.

As a site proposed for testing vaccines in humans, we requested and were given approval for evaluating different vaccine candidates for safety and effectiveness. Two whole coronavirus

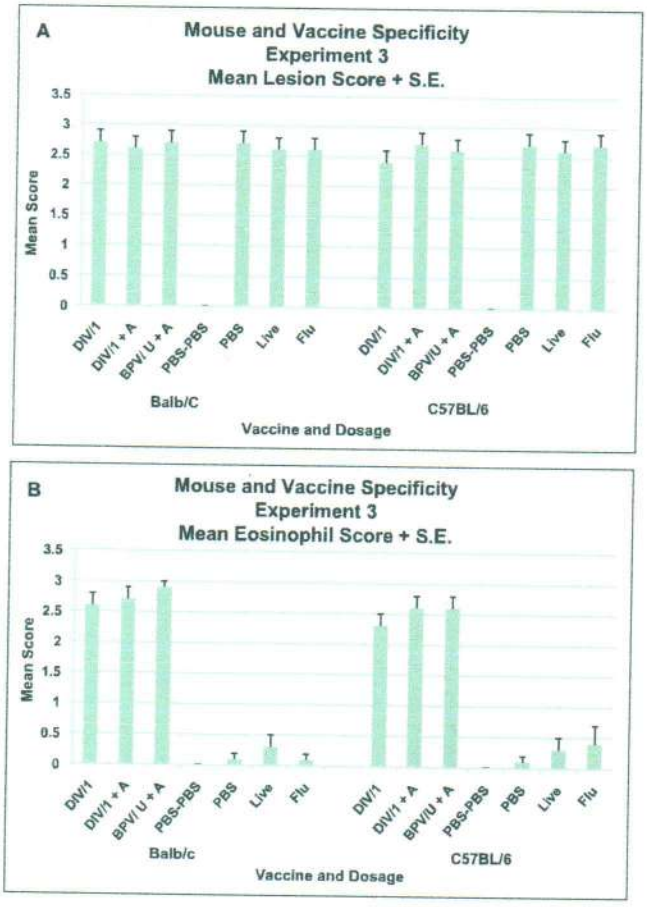

Figure 7. Mouse and Vaccine Specificity, Experiment 3. Serum neutralizing (neut) antibody and lung virus titers for each vaccine dosage group. A. Geometric mean serum antibody titer and standard error of the mean (S.E.) on day 56 for each vaccine dosage group for each mouse strain (Balb/c or C57BL/6). Five mice per group given 0.1 ml of vaccine intramuscularly on days 0 and 28. B. Geometric mean virus titer (log_{10} $TCID_{50}$/g) and standard error of the mean (S.E.) in lungs on day 58 (two days after SARS-CoV challenge for each vaccine dosage group for each mouse strain. Seven to eight mice per group. Vaccines: Double inactivated whole virus, (DIV), β propiolactone inactivated whole virus (BPV), with alum (+A). Analyses: A. GMT for highest DIV dosage without alum greater for Balb/c than C57BL/6 $p = .008$ but not for alum $p > .05$. GMT for the BPV vaccine and live virus were not different for the two strains $p > .05$. B. GMT for PBS control mice were not different $p > .05$. GMT for DIV without alum and BPV with alum greater for C57BL/6 than Balb/c $p = .004$.
doi:10.1371/journal.pone.0035421.g007

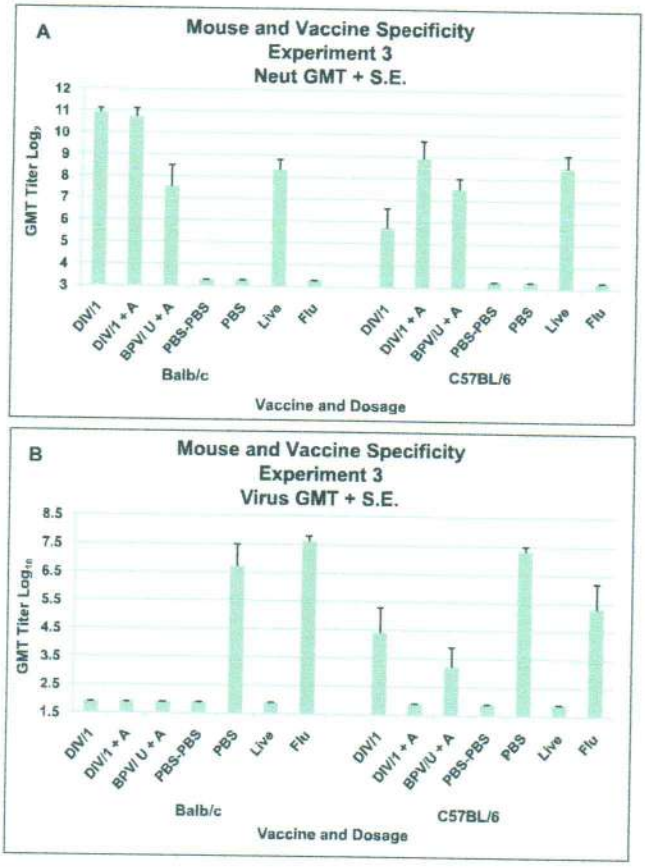

Figure 8. Mouse and Vaccine Specificity, Experiment 3. Mean lung cellular infiltration/lesion pathology and percent eosinophils in infiltrates for each vaccine dosage group for each mouse strain (Balb/c or C57BL/6) two days after challenge with SARS-CoV. A. Mean lesion score and standard error of the mean (S.E.) for each vaccine dosage group. Scores are mean of scores for seven to eight mice per group. Scoring 0 - no definite pathology, 1 - mild peribronchiole and perivascular cellular infiltration, 2 - moderate peribronchiole and perivascular cellular infiltration, 3 - severe peribronchiole and perivascular cellular infiltration with thickening of alveolar walls, alveolar infiltration and bronchiole epithelial cell necrosis and debris in the lumen. Ten to 20 microscopy fields were scored for each mouse lung. B. Mean score and standard error of the mean (S.E.) for eosinophils as percent of infiltrating cells for each vaccine dosage group. Scores are mean of scores for seven to eight mice per group. Scoring: 0 - <5% of cells, 1 - 5–10% of cells, 2 - 10–20% of cells, 3 - >20% of cells. Ten to 20 microscopy fields were scored for each mouse lung. Analyses: A. Mean lesion scores were not different $p > .05$. B. Mean eosinophil scores were different $p < .001$. Mean scores for vaccine groups greater than non-vaccine groups for Balb/c and C57BL/6 $p < .001$ for all comparisons. Mean eosinophil scores for the same groups not different for Balb/c and C57BL/6 $p > .05$.
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vaccines, one rDNA-expressed S protein vaccine and a VLP vaccine prepared by us were evaluated in a Balb/c mouse model, initially described by others, of SARS-CoV [46,47]. The concern for an occurrence of lung immunopathology on challenge of mice vaccinated with an inactivated virus vaccine, as reported by Haagmans, et al. for ferrets and nonhuman primates, was seen by us after challenge of mice vaccinated with a SARS VLP vaccine [20]. This finding was duplicated in an experiment reported here and was also seen in mice vaccinated with a range of dosages of a double-inactivated whole virus vaccine (DIV) and an rDNA S protein vaccine (SV) although the immunopathologic reaction appeared reduced among animals given the S protein vaccine when compared to those given the whole virus vaccine. In later experiments, these findings were confirmed and the vaccine utilized by Haagmans, et al. was also shown to induce the immunopathology in mice. Thus, all four vaccines evaluated induced the immunopathology; however, all four also induced neutralizing antibody and protection against infection when compared to control challenged animals.

The immunopathology in all experiments in the present study occurred in the absence of detectable virus in lungs of mice two days after challenge with infectious virus. In two experiments, a live virus group subsequently challenged with live virus was included. These challenged animals also exhibited similar histopathologic changes after challenge although no infectious virus was detected in lungs on day two; however, in the latter case, the infiltrates were nearly 100%

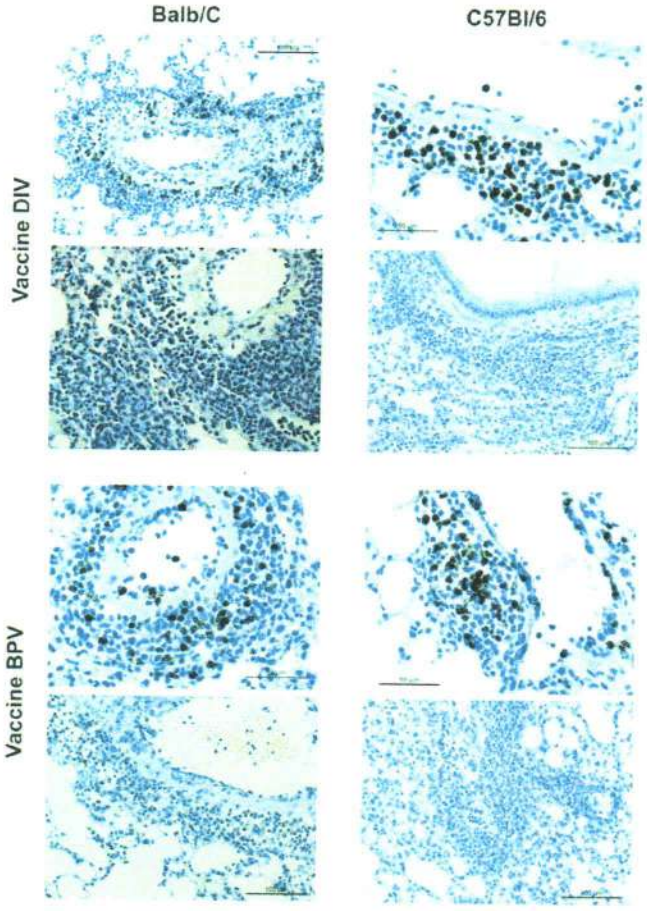

Figure 9. Photomicrographs of Lung Tissue. Representative photomicrographs of lung tissue two days after challenge of Balb/c and C57BL/6 mice that had previously been given a SARS-CoV vaccine. Lung sections were separately stained with H&E (pink and blue micrographs) or the immunohistochemical stain for eosinophil major basic protein (blue and brown micrographs). Balb/c mice lung sections are in the left column and C57BL/6 are in the right column; doubly inactivated whole virus vaccine is in the upper four panels and those from mice given the β propiolactone inactivated whole virus vaccine are in the lower four panels. Pathologic changes observed (inflammatory infiltrates) are similar in Balb/c and C57BL/6 and eosinophils are prominent in both groups.
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monocytes and lymphocytes without the eosinophil component seen in the vaccinated challenged animals. In a separate test to assess the effects of the challenge inoculum, mice were given an IN challenge with 10^8TCID$_{50}$ of inactivated whole SARS-CoV. Lungs of these animals revealed minimal or no histopathologic damage (data not shown). These findings suggest that virus replication probably occurred early after challenge, including in animals given live CoV earlier, and is required for development of pathology, including for the immunopathology. Infection would have been transient, below the limit of detection two days after challenge, or neutralized in lung homogenates before testing for virus.. Nevertheless, the Th2-type immunopathology pattern was seen only in animals given an inactivated vaccine earlier.

During the course of these experiments, a report appeared describing a similar immunopathologic-type reaction with prominent eosinophils in SARS-CoV challenged Balb/c mice that had been given Venezuelan equine encephalitis (VEE) vector containing the SARS nucleocapsid protein gene [18]. Those challenged animals exhibited infection similar to unvaccinated animals as well as Th2-type immunopathology. A similar experiment with a VEE vector containing only the S gene exhibited protection against infection and no immunopathology. More recently, this group has reported immunopathology with prominent eosinophil infiltration after SARS-CoV challenge in Balb/c mice vaccinated with the same double-inactivated whole virus vaccine used in our experiments [28]. They attribute the immunopathologic reaction following these SARS-CoV vaccinations to presence of the nucleocapsid protein (N) in the vaccine.

In another report, vaccinia was used as a vector vaccine for immunizing Balb/c mice with each of the SARS-CoV structural proteins (N, S, membrane, and envelope) and then challenged with SARS-CoV [21]. Virus infection was present in all groups after challenge but reduced in the S vector vaccine group. Histopathology scores were high for the N containing vector group and low for the S containing group and for the vehicle control group. Eosinophilic infiltrates and IL-5 were increased in the N vaccine group but only IL-5 was increased in the S vaccine group.

To be certain the Th2 type immunopathology was elicited by the S protein vaccine in our studies and in hopes a greater immune response would result from higher dosages of the vaccine and induce greater protection against infection as well as reduce or prevent the immunopathology, our experiment 2 used up to 9 μg of the S protein for immunization. While increased titers of serum antibody were induced and no virus was detected day two after challenge in most animals, the Th2-type immunopathology occurred after challenge, and the immunopathology seen earlier after vaccination with the DI whole virus vaccine was seen again. This experiment also included the whole virus vaccine tested earlier in ferrets and nonhuman primates where the Th2-type immunopathology was initially seen. That vaccine, the BPV in this report, exhibited a pattern of antibody response, protection against infection and occurrence of immunopathology after challenge similar to the DI whole virus vaccine (DIV).

A final experiment was conducted to evaluate specificity. The Balb/c mouse was compared to C57BL/6 mice which do not exhibit the Th2 response bias known to occur in Balb/c mice. C57BL/6 mice in that same experiment exhibited results on challenge similar to those seen in Balb/c mice. Challenge of animals given prior influenza vaccine were infected and exhibited histopathologic damage similar to animals given PBS earlier; neither group exhibited the eosinophil infiltrations seen in animals given a SARS-CoV vaccine.

In these various experiments alum was used as an adjuvant and this adjuvant is known to promote a Th2 type bias to immune responses [48]. However, the immunopathology seen in vaccinated-challenged animals also occurred in animals given vaccine without alum. In an effort to determine whether an adjuvant that induced a bias for a Th1-type response would protect and prevent the immunopathology, we initiated an experiment where the DI PBS suspended vaccine was adjuvanted with Freund's complete adjuvant, a Th1-type adjuvant. However, this experiment was aborted by the September, 2008, Hurricane Ike induced flood of Galveston, Texas. An experiment with a SARS-CoV whole virus vaccine with and without GlaxoSmithKline (GSK) adjuvant ASO1 in hamsters has been reported [25]. This adjuvant is thought to induce Th1-type immune responses [49]. The authors indicate no lung immunopathology was seen among animals after challenge, including the group given vaccine without adjuvant; however, whether the hamster model could develop a Th2-type immunopathology is uncertain. Finally, a number of other studies of vaccines in animal model systems have been reported but presence or absence of immunopathology after challenge was not reported.

Table 2. Summary of Reported Protection and Immunopathology in Animal Model Studies with SARS Coronavirus Vaccines.

Animal Model	Vaccine[1]	Protection[2]	Immunopathology[3]
Mice	Whole virus[tr]		
	w alum	Yes	Yes
	Whole virus[25,tr]		
	w alum	Yes	Yes
	wo alum	Yes	Yes
	VLP[17,tr]		
	w alum	Yes	Yes
	wo alum	Yes	Yes
	S Protein[tr]		
	w alum	Yes	Yes
	wo alum	Yes	Yes
	VEE Vector[15]		
	for N protein	No	Yes
	for S protein	Yes	No
	Vaccinia vector[18]		
	for N protein	No	Yes
	for S protein	Yes	?No
Ferrets	Whole virus[11]		
	w alum	Yes	Yes
Nonhuman Primate[4]	Whole virus[11]		
	w alum	Yes	Yes
Hamsters	Whole virus[22]		
	w ASO1	Yes	No

[1]Reference for each indicated; tr = this report; w = with, wo = without.
[2]Protection against infection (reduced lung virus after challenge).
[3]Th2-type immunopathology as indicated by cellular infiltrates with prominence of eosinophils.
[4]Cynomolgus monkeys.
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A summary of the SARS-CoV vaccine evaluations in animal models (including the current report) that indicated an evaluation for immunopathology after challenge is presented in Table 2. As noted all vaccines containing S protein induced protection against infection while the studies with VEE and vaccinia vector containing the N protein gene only did not. Also shown is that a Th2-type immunopathology was seen after challenge of all vaccinated animals when evaluation for immunopathology was reported except the study in hamsters with a GSK whole virus vaccine. Thus, inactivated whole virus vaccines whether inactivated with formalin or beta propiolactone and whether given with our without alum adjuvant exhibited a Th2-type immunopathologic in lungs after challenge. As indicated, two reports attributed the immunopathology to presence of the N protein in the vaccine; however, we found the same immunopathologic reaction in animals given S protein vaccine only, although it appeared to be of lesser intensity. Thus, a Th2-type immunopathologic reaction on challenge of vaccinated animals has occurred in three of four animal models (not in hamsters) including two different inbred mouse strains with four different types of SARS-CoV vaccines with and without alum adjuvant. An inactivated vaccine preparation that does not induce this result in mice, ferrets and nonhuman primates has not been reported.

This combined experience provides concern for trials with SARS-CoV vaccines in humans. Clinical trials with SARS coronavirus vaccines have been conducted and reported to induce antibody responses and to be "safe" [29,30]. However, the evidence for safety is for a short period of observation. The concern arising from the present report is for an immunopathologic reaction occurring among vaccinated individuals on exposure to infectious SARS-CoV, the basis for developing a vaccine for SARS. Additional safety concerns relate to effectiveness and safety against antigenic variants of SARS-CoV and for safety of vaccinated persons exposed to other coronaviruses, particularly those of the type 2 group. Our study with a VLP SARS vaccine contained the N protein of mouse hepatitis virus and Bolles, et al., reported the immunopathology in mice occurs for heterologous Gp2b CoV vaccines after challenge [25]. This concern emanates from the proposal that the N protein may be the dominant antigen provoking the immunopathologic reaction.

Because of well documented severity of the respiratory disease among infants given an inactivated RSV vaccine and subsequently infected with RSV that is considered to be attributable to a Th2-type immunopathologic reaction and a large number of studies in the Balb/c mouse model that have described and elucidated many components of the immunopathologic reaction to RSV vaccines, the similarity to the SARS-CoV vaccine evaluations in Balb/c mice supports caution for clinical vaccine trials with SARS-CoV vaccines in humans. Of interest are the similar occurrences in C57BL/6 mice and in ferrets and nonhuman primates that provide alternative models for elucidating vaccine-induced mechanisms for occurrences of Th2 immunopathologic reactions after infection. As indicated, strong animal model evidence indicates expression of the N protein by SARS-CoV vector vaccines can induce sensitization leading to a Th2–type immunopathology with infection. In contrast to our results, those studies did not find clear evidence of the Th2 type immunopathology on challenge of mice given a vector vaccine for the S protein. The finding of a Th-2-type pathology in our studies in animals immunized with an rDNA-produced S protein is unequivocal. In this regard, animal model studies with FIPV in cats and RSV in mice have indicated that viral surface proteins may be the sensitizing protein of inactivated vaccines for immunopathology with infection [32,45]. This suggests that presentation of the S protein in a vector format may direct immune responses in a different way so that sensitization does not occur.

Limitations of the present studies include their performance in mice only and uncertainty of the relevance of rodent models to SARS-CoV vaccines in humans. Additionally, a more intense study for virus replication including quantitative RT-PCR assays might have confirmed the probability that virus replication is required for induction of the immunopathology after vaccination. Evaluations of mechanisms for the immunopathology, including immunoglobulin and cytokine responses to vaccines and tests for antigen-antibody complexes in tissues exhibiting the reaction, could have strengthened the Th2-type immunopathology finding. Finally, a successful study with a Th1-type adjuvant that did not exhibit the Th2 pathology after challenge would have confirmed a Th2 bias to immune responses as well as provide a potential safe vaccination approach for SARS.

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Author Contributions

Conceived and designed the experiments: RBC CJP C-TT. Performed the experiments: C-TT ES NI-Y PCN TG. Analyzed the data: RLA RBC C-TT. Contributed reagents/materials/analysis tools: RBC C-TT RLA ES. Wrote the paper: RBC C-TT ES.

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Informed consent disclosure to vaccine trial subjects of risk of COVID-19 vaccines worsening clinical disease

Nuremberg Code Violation

Abstract

Aims of the study: Patient comprehension is a critical part of meeting medical ethics standards of informed consent in study designs. The aim of the study was to determine if sufficient literature exists to require clinicians to disclose the specific risk that COVID-19 vaccines could worsen disease upon exposure to challenge or circulating virus.

Methods used to conduct the study: Published literature was reviewed to identify preclinical and clinical evidence that COVID-19 vaccines could worsen disease upon exposure to challenge or circulating virus. Clinical trial protocols for COVID-19 vaccines were reviewed to determine if risks were properly disclosed.

Results of the study: COVID-19 vaccines designed to elicit neutralising antibodies may sensitise vaccine recipients to more severe disease than if they were not vaccinated. Vaccines for SARS, MERS and RSV have never been approved, and the data generated in the development and testing of these vaccines suggest a serious mechanistic concern: that vaccines designed empirically using the traditional approach (consisting of the unmodified or minimally modified coronavirus viral spike to elicit neutralising antibodies), be they composed of protein, viral vector, DNA or RNA and irrespective of delivery method, may worsen COVID-19 disease via antibody-dependent enhancement (ADE). This risk is sufficiently obscured in clinical trial protocols and consent forms for ongoing COVID-19 vaccine trials that adequate patient comprehension of this risk is unlikely to occur, obviating truly informed consent by subjects in these trials.

Conclusions drawn from the study and clinical implications: The specific and significant COVID-19 risk of ADE should have been and should be prominently and independently disclosed to research subjects currently in vaccine trials, as well as those being recruited for the trials and future patients after vaccine approval, in order to meet the medical ethics standard of patient comprehension for informed consent.

1 | THE RISK OF ADE IN COVID-19 VACCINES IS NON-THEORETICAL AND COMPELLING

Vaccine-elicited enhancement of disease was previously observed in human subjects with vaccines for respiratory syncytial virus (RSV), dengue virus and measles.[1] Vaccine-elicited enhancement of disease was also observed with the SARS and MERS viruses and with feline coronavirus, which are closely related to SARS-CoV-2, the causative pathogen of COVID-19 disease. The immune mechanisms of this enhancement have invariably involved antibodies, from direct antibody-dependent enhancement, to immune complex formation by antibodies, albeit accompanied by various coordinated cellular responses, such as Th2 T-cell skewing.[2-7] Notably, both neutralising and non-neutralising antibodies have been implicated. A recent study revealed IgG-mediated acute lung injury in vivo in macaques infected with SARS that correlated with a vaccine-elicited, neutralising antibody response.[8] Inflammation and tissue damage in the lung in this animal model recapitulated the inflammation and tissue damage in the lungs of SARS-infected patients who succumbed to the disease. The time course was also similar, with the worst damage occurring in delayed fashion in synchrony with ramping up of the immune response. Remarkably, neutralising antibodies controlled the virus in the animal, but then would precipitate a severe, tissue-damaging, inflammatory response in the lung. This is a similar profile to immune complex-mediated disease seen with RSV vaccines in the past, wherein vaccinees succumbed to fatal enhanced RSV disease because of the formation of antibody-virus immune complexes that precipitated harmful, inflammatory immune responses. It is also similar to the clinical course of COVID-19 patients, in whom severe COVID-19 disease is associated with the development of anti-SARS-CoV-2 serum antibodies,[9] with titres correlating directly with the severity of disease.[10] Conversely, subjects who recover quickly may have low or no anti-SARS-CoV-2 serum antibodies.[11]

The elicitation of antibodies, specifically neutralising antibodies, is the goal of nearly every current SARS-CoV-2 vaccine candidate. The prior evidence that vaccine-elicited, antibody-dependent enhancement (ADE) of disease is likely to occur to some degree with COVID-19 vaccines is vertically consistent from controlled SARS studies in primates to clinical observations in SARS and COVID-19. Thus, a finite, non-theoretical risk is evident in the medical literature that vaccine candidates composed of the SARS-CoV-2 viral spike and eliciting anti-SARS-CoV-2 antibodies, be they neutralising or not, place vaccinees at higher risk for more severe COVID-19 disease when they encounter circulating viruses. Indeed, studies in mice of prior SARS vaccines revealed this exact phenotype, with four human vaccine candidates eliciting neutralising antibodies and protecting against SARS challenge, but viral re-challenge of thus vaccinated animals resulting

in immunopathologic lung disease.[5] Independently, SARS/MERS vaccine candidates, commonly exhibited ADE associated with high inflammatory morbidity in preclinical models, obstructing their advancement to the clinic.[4,12] SARS ADE of both disease in non-human primates and viral infection of cells in vitro was clearly mapped to specific antibody-targeted SARS viral spike epitopes.[6] This phenomenon was consistent across a variety of vaccine platforms, including DNA, vector primes and virus-like particles (VLP), irrespective of inoculation method (oral, intramuscular, subcutaneous, etc). An unknown variable is how long this tissue damage lasts, possibly resulting in permanent morbidity (eg, diabetes from pancreatic damage[7]).

Current data on COVID-19 vaccines is limited, but does not so far reveal evidence of ADE of disease. Non-human primate studies of Moderna's mRNA-1273 vaccine showed excellent protection, with no detectable immunopathology.[13] Phase 1 trials of several vaccines have not reported any immunopathology in subjects administered the candidate vaccines. However, these subjects were unlikely to have yet encountered circulating virus.[14] Nevertheless, all preclinical studies to date have been performed with the Wuhan or closely related strains of the virus, while a mutant D614G virus is now the most prevalent circulating form. Several observations suggest that this alternative form may be antigenically distinct from the Wuhan derived strain, not so much in composition, but in conformation of the viral spike and exposure of neutralisation epitopes.[15-18] Similarly, Phase 1 and 2 clinical trials of vaccine candidates have only been designed around immunogenicity as an efficacy end point and have not been designed to capture exposure of subjects to circulating virus after vaccination, which is when ADE/immunopathology is designed to occur. Thus, the absence of ADE evidence in COVID-19 vaccine data so far does not absolve investigators from disclosing the risk of enhanced disease to vaccine trial participants, and it remains a realistic, non-theoretical risk to the subjects.

2 | CHALLENGES TO INFORMED CONSENT FOR COVID-19 VACCINE STUDIES

Informed consent procedures for vaccine trials commonly include disclosure of very minor risks such as injection site reactions, rare risks from past, *unrelated* vaccines/viruses, such as Guillain-Barre syndrome for swine flu (interest in which is likely behind the interest in Astra Zeneca's recent vaccine transverse myelitis event) and generic statements about the risk of idiosyncratic systemic adverse events and death. Specific risks to research participants derived from biological mechanism are rarely included, often because of ambiguity about their applicability.[19]

Signed consent forms from the COVID-19 vaccine trials are not publicly available because of privacy concerns. They also vary from clinical site to clinical site, and sample consent forms on which they are based are not required to be disclosed until after the trial is over, if at all. However, these consent forms are usually very similar in content to the "Risks to participants" section of the trial protocols, which have been released publicly by Pfizer, Moderna and Johnson & Johnson for their COVID-19 vaccine trials ([20] & Supplement). As these three vaccines are representative of the diversity of vaccines being tested, it is very likely that the consent form inferred from these protocols is similar or identical to those from any and all of the vaccine trials currently underway. All three protocols mention the risk of disease enhancement by the vaccine, but all three list this risk last or next to last in the list of risks, after risks from the Ad26-Cov2 vector, adenovirus vectors in general, risks of vaccination in general, risks for pregnancy and birth control (which are said to be "unknown"), risks of blood draws and risks from collection of nasal swab samples (for the Johnson and Johnson vaccine), after allergy, fainting, local site injection reaction, general systemic adverse reactions and laboratory abnormalities for the Moderna vaccine and after local site injection reactions and general systemic adverse events for the Pfizer vaccine. In addition, both Moderna and Johnson and Johnson term the risk of vaccine-elicited disease enhancement as "theoretical." Finally, in citing the risk, Pfizer and Moderna note prior evidence of vaccine-elicited disease enhancement with RSV and dengue, as well as feline coronavirus (Pfizer) and measles (Moderna), however, SARS and MERS are not mentioned. Johnson and Johnson discusses SARS and MERS, but make an unusual scientific argument that vaccine-elicited disease enhancement is because of non-neutralising antibodies and Th2-skewed cellular responses and that Ad26 vaccination does not exhibit this profile.Blank consent forms for AstraZeneca and Johnson and Johnson are also available online at https://restoringtrials.org/2020/09/18/covid19trialprotocolandstudydocs/, and while the AstraZeneca form clearly discloses the specific risk of ADE, the disclosure is listed last among risks only in an attached information sheet. In all, the evidence from the Pfizer, Moderna and Johnson & Johnson protocols for their COVID-19 vaccine trials and the sample consent forms, when contrasted with the evidence for antibody-dependent enhancement of disease presented by this report and widely available to any skilled practitioner in the field, establishes that patient comprehension of the specific risk that receiving the COVID-19 vaccine could convert a subject from someone who experiences mild disease to someone who experiences severe disease, lasting morbidity or even death is unlikely to be achieved by the informed consent procedures planned for these clinical trials.

Medical ethics standards required that, given the extent of evidence in the medical literature reviewed above, the risk of ADE should be clearly and emphatically distinguished in the informed consent from risks observed *rarely* as well as the more obvious risk of lack of efficacy, which is unrelated to the specific risk of ADE. Based on the published literature, it should have been obvious to any skilled medical practitioner in 2019 that there is a significant risk to vaccine research subjects that they may experience severe disease once vaccinated, while they might only have experienced a mild, self-limited disease if not vaccinated. The consent should also clearly distinguish the specific risk of worsened COVID-19 disease from generic statements about risk of death and generic risk of lack of efficacy of the vaccine.

3 | CONCLUSION

Given the strong evidence that ADE is a non-theoretical and compelling risk for COVID-19 vaccines and the "laundry list" nature of informed consents, disclosure of the specific risk of worsened COVID-19 disease from vaccination calls for a specific, separate, informed consent form and demonstration of patient comprehension in order to meet medical ethics standards. The informed consent process for ongoing COVID-19 vaccine trials does not appear to meet this standard. While the COVID-19 global health emergency justifies accelerated vaccine trials of candidates with known liabilities, such an acceleration is not inconsistent with additional attention paid to heightened informed consent procedures specific to COVID-19 vaccine risks.

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DISCLOSURE

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

TC and RV conceived this commentary. TC wrote the manuscript. RV edited and approved the manuscript.

DATA AVAILABILITY STATEMENT

All data referenced in this report have been published in peer-reviewed literature or are available on the World Wide Web/Internet at the URL's indicated in the References section. Therefore, all data referenced in this report are publicly available in widely available data repositories.

Timothy Cardozo[1]
Ronald Veazey[2]

[1]*Department of Biochemistry and Molecular Pharmacology, NYU Langone Health, New York, NY, USA*
[2]*Division of Comparative Pathology, Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, Tulane National Primate Research Center, Covington, LA, USA*

Correspondence
Timothy Cardozo, Department of Biochemistry and Molecular Pharmacology, NYU Langone Health, 550 First Avenue, MSB 222, New York, NY 10016, USA.
Email: cardot01@nyumc.org

ORCID

Timothy Cardozo https://orcid.org/0000-0002-0643-4497

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Morton, IL Police Department

Bethanie Ruder

FOIA Officer

375 Birchwood Street

Morton, Illinois 61550

Dear Bethanie Ruder,

Hello.

I am contacting this office in accordance to the Freedom of Information Act (FOIA), 5 ILCS 140.

I am requesting copies of "all records, reports, forms, writings, letters, memoranda, books, papers, maps, photographs, microfilms, cards, tapes, recordings, electronic data processing records, electronic communications, recorded information and all other documentary materials pertaining to the transaction of public business, regardless of physical form or characteristics, having been prepared by or for, or having been or being used by, received by, in the possession" of the Morton, Illinois Police Department regarding Michael D. Jackson on 31 December, 2020 A.D. and/or thereafter.

Thank you for your kind assistance.

America bless God again.

Michael D. Jackson R.N.

Michael D. Jackson R.N.

9 January, 2021 A.D.

CAD

Event Report

Event ID: **20-183027** Call Ref #: 958 Date/Time Received: 12/31/20 14:45:04

Rpt #:
Call Source: PHONE
Prime Unit: MO15 ROWE, AARON C

Services Involved: LAW

Location: **825 DETROIT AV**
X-ST: *W EDGEWOOD CT*
W HAZELWOOD
Business: CEFCU MO

Jur: CAD Service: LAW Agency: MPD
St/Beat: MO1 District: RA: MO11
Phone: (309) 633-7120 GP: MO1

Nature: **ALL OTHER DISORDERLY** Alarm Lvl: 1 Priority: 5 Medical Priority:

Reclassified Nature:

Caller: [redacted]
Addr: [redacted] Phone: [redacted] Alarm:
Alarm Type:

Vehicle #: St: Report Only: No Race: Sex: Age:

Call Taker: TROTTERR Console: TC06

Geo-Verified Addr.: Yes Nature Summary Code: Disposition: 40 Close Comments:

Notes: MALE REFUSING TO WEAR A MASK AND NOW REFUSING TO LEAVE [12/31/20 14:45:47 TROTTERR]
JACKSON,MICHAEL D [redacted] 12/31/20 14:50:45 POTTERJ]

Times

		Time From Call Received		
Call Received:	12/31/20 14:45:04			
Call Routed:	12/31/20 14:45:47	000:00:43	Unit Reaction:	000:01:32 *(1st Dispatch to 1st Arrive)*
Call Take Finished:	12/31/20 14:45:47	000:00:43	En-Route:	000:00:18 *(1st Dispatch to 1st En-Route)*
1st Dispatch:	12/31/20 14:46:11	000:01:07 *(Time Held)*	On-Scene:	000:18:03 *(1st Arrive to Last Clear)*
1st En-Route:	12/31/20 14:46:29	000:01:25		
1st Arrive:	12/31/20 14:47:43	000:02:39 *(Reaction Time)*		
Last Clear:	12/31/20 15:05:46	000:20:42		

Radio Log

Unit	Empl ID	Type	Description	Time Stamp	Comments (may truncate in portrait)	Close Code	User
MO15	12015	D	Dispatched	12/31/20 14:46:11	Stat/Beat: MO1		POTTERJ
MO3	1233	D	Dispatched	12/31/20 14:46:11	Stat/Beat: MOR		POTTERJ
MO15	12015	E	En-Route	12/31/20 14:46:29			POTTERJ
MO3	1233	E	En-Route	12/31/20 14:46:29			POTTERJ
MO15	12015	A	Arrived	12/31/20 14:47:43			POTTERJ
MO3	1233	A	Arrived	12/31/20 14:47:43			POTTERJ
MO15	12015	C	Cleared	12/31/20 15:01:13		40	WARDA
MO3	1233	C	Cleared	12/31/20 15:05:46		07	WARDA