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PURIFICATION OF INFLUENZA VIRUS
POLYPEPTIDE ANTIGENS AND STUDIES
OF THEIR IMMUNOGENICITY AND TOXICITY

ANNUAL PROGRESS REPORT

by

Edwin D. Kilbourne, M. D.
Doris J. Bucher, Ph. D.

September, 1976

(For the period 31 July 1975 - 1 August 1976)

Supported by
U. S. Army Medical Research and Development Command
Office of the Surgeon General, Washington, D. C.   20315

Contract No. DADA17-69-C-9137

Mount Sinai School of Medicine
of the City University of New York 10029

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Continued development of an "infection-permissive" neuraminidase-specific vaccine has proceeded. Such a vaccine may be produced either by tailor-making a recombinant virus containing the neuraminidase of the current subtype of influenza virus coupled with an irrelevant hemagglutinin or by isolation of the neuraminidase directly from the virus.

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20.   ABSTRACT - (Continued)

Vaccine trials have compared the immune response of a neuraminidase-specific vaccine employing the recombinant X-38 (Heq1N2) with that of a conventional bispecific vaccine, X-37 (H3N2). A superior antibody response to neuraminidase was observed when the enzyme was coupled with the "irrelevant" hemagglutinin in the X-38 vaccine as compared with the conventional vaccine. An unexpected finding was the induction of anti-H3 heterotypic antibody by the X-38 vaccine possessing the "irrelevant" hemagglutinin. However, these antibodies are apparently non-neutralizing.

A neuraminidase-specific vaccine for swine influenza (X-54a) has been produced as an antigenic hybrid containing the swine influenza virus neuraminidase combined with an "irrelevant" hemagglutinin (Heq1 N1sw).

Preliminary trials of immunization of man with isolated neuraminidase antigen revealed: 1) no toxicity, 2) adequate immunogenicity and 3) contamination of the preparation with viral hemagglutinin.

Techniques for viral protein purification have been improved and have shown that hemagglutinin as a dimer is immunogenic.

FOREWORD

In conducting the research described in this report, the
investigator(s) adhered to the "Guide for Laboratory Animal
Facilities and Care," as promulgated by the Committee on
the Guide for Laboratory Animal Resources, National Academy
of Science - National Research Council.

SUMMARY

Much of our report deals with efforts directed towards the continued development of an "infection-permissive" neuraminidase-specific vaccine. Such a vaccine may be produced either by tailor-making a recombinant virus containing the neuraminidase of the current subtype of influenza virus coupled with an irrelevant hemagglutinin or by purification of the neuraminidase directly from the vaccine.

Vaccine trials have been conducted which compare the immune response of a neuraminidase-specific vaccine employing the recombinant X-38 (Heq1N2) with that of a conventional bispecific vaccine, X-37 (H3N2). Three groups of college students received either conventional A/England/42/72 (H3N2) vaccine (X-37), neuraminidase monospecific vaccine (Heq1N2) (X-38), or a placebo injection. An interesting finding was the superior antibody response to neuraminidase observed when the enzyme was coupled with the "ir-relevant" hemagglutinin in the X-38 vaccine as com-pared with the X-37 vaccine. The homotypic hemag-glutination inhibition response to vaccine hemag-glutinin occurred with almost the same frequency among vaccinees receiving either X-37 or X-38 vaccines, with a lesser degree of response for X-38. An unexpected finding was the significant induction of anti-H3 anti-body by the X-38 vaccine possessing the "irrelevant" hemagglutinin. However, unlike the usual antibody response to hemagglutinin, these antibodies are apparently non-neutralizing.

Neuraminidase-specific immunization for protection against swine influenza will also be possible using an antigenic hybrid (X-54a) containing the swine influenza virus neuraminidase combined with an "ir-relevant" hemagglutinin (Heq1 N1sw). This virus is

already under experimental production as a vaccine
by Lederle Laboratories for use in field trials.

Preliminary results are reported for a neura-
minidase-specific vaccine utilizing the enzyme
purified from a commercial lot of vaccine. Follow-
ing disruption of the virus, the neuraminidase was
purified by adsorption and elution from an affinity
column. The major modification required for pro-
duction of the neuraminidase for vaccine purposes
was the substitution of Tween 80 for Triton X-100
as a stabilizing agent, permitting use in human
subjects. An extra benefit of the preparation pro-
cedure was a significant lowering of contaminating
endotoxin. Immune response to the purified neura-
minidase was good in human subjects when the dose
was adjusted to the equivalent enzymatic activity
of the starting vaccine preparation. However, con-
siderable hemagglutinin contaminated the enzyme
preparation as could be seen by the hemagglutination
inhibition titers, particularly at the higher doses.
Future neuraminidase vaccine preparations will
require harsher conditions for disruption of the virus
and the use of recombinant viruses containing detergent
sensitive hemagglutinins.

Chromatographic isolation of the hemagglutinin,
neuraminidase and other viral polypeptides has con-
tinued with the aim of producing sufficient quantities
of purified materials for studies of the immunogeni-
city and biochemistry of these proteins. A cross-
linked agarose media permits chromatography of larger
quantities of viral protein at the first step of gel
filtration. A new chromatographic technique has been
developed for the removal of the detergent SDS from
protein preparations and their subsequent renaturation
without the significant losses of material incurred
by earlier techniques.

One difficulty of the SDS gel filtration has
been the lack of immunogenicity of the purified
hemagglutinin polypeptides. Our chromatographically
purified hemagglutinin polypeptides as HA_1, HA_2 or
as a mixture of HA and NP from SDS gel filtration
have been remarkably non-immunogenic. We are now
able to achieve a good immune response with a hemag-
glutinin fraction which elutes from SDS gel filtration
apparently as a dimer when the starting viral prepar-
ation is disrupted with a low level of SDS to protein.

Our pyrogenicity studies with Dr. Atkins are
underway. Commercial vaccine preparations and our
own sucrose gradient viral preparations have been
examined and both are remarkable for their low level
of pyrogenic response in rabbits. Preparations of
swine flu vaccine are also being examined with the aim
of relating the pyrogenic response in rabbits with
reactogenicity in humans.

Table of Contents

Comparative efficacy of neuraminidase-specific and
conventional influenza virus vaccines in the induction
of anti-neuraminidase antibody in man. (Preliminary
findings were summarized in progress report for 1974-
75). (J. Infectious Diseases in press) (E. D. Kilbourne)

Groups of college students (30-39 per group)
received either conventional A/England/42/72 (H3N2)
vaccine (X-37), or an antigenically hybrid (Heq1N2)
vaccine (X-38) containing the same neuraminidase
(Figure 1), and effectively neuraminidase-monospecific,
or a placebo injection. The vaccines contained 798
and 643 CCA units respectively per dose and equivalent
immunogenic units of N2 as defined in antigenic ex-
tinction tests in rabbits (Table 1). All subjects had
pre-immunization anti-N2 antibody and mean initial
titers were comparable in both vaccine groups (1:56).
Homotypic hemagglutination-inhibition (HI) response
to vaccine hemagglutinin occurred with a frequency
of 72% in X-37 and 77% in X-38 vaccinees but was less
in magnitude in the X-38 than in the X-37 group
(Table 2). Significant anti-N2 (NI) response was
observed in 25% of X-37 and 69% of X-38 vaccinees
(Table 2) - an unexpected finding. Mean NI response
was 2 fold greater in the X-38 group. Another un-
anticipated finding was the occurrence of heterotypic
HI response (i.e., versus H3) in 56% of those receiving
X-38 vaccine (Table 3). In preliminary plaque in-
hibition titrations this heterotypic antibody did not
have neutralizing activity. Testing of NI response
with earlier neuraminidase antigens demonstrated
"original antigenic sin" from earlier priming involving
in one instance also the N1 neuraminidase (Figure 2).
The superiority of the "neuraminidase-specific" X-38
(Heq1N2) vaccine as an immunogen for anti-neuraminidase
antibody may reflect different processing of the N2
neuraminidase when it is associated with hemagglutinin
antigen to which the study population is inexperienced.
(see below)

A/Eng/42/72 (H3N2) (MRC-2)   A/Equine/Prague/1/56 (Heq1 Neq1)   A/PR/8/34 (HON1)

X-38
(Heq1 N2)
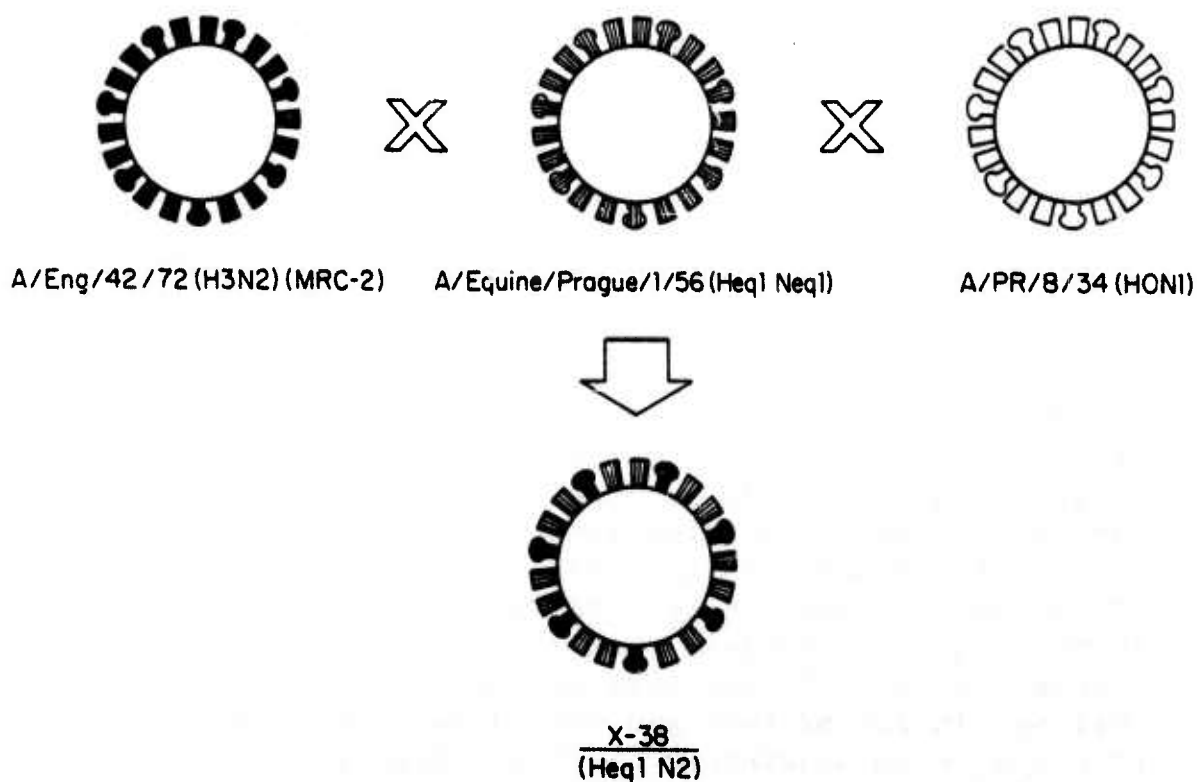
Figure 1. Derivation of antigenically hybrid recom-
binant virus (X-38) employed as neuraminidase-specific
vaccine. Following mixed infection of the chick embryo
allantoic sac with the three parental viruses shown,
recombinant X-38, bearing Heq1 hemagglutinin and $N2_{Eng}$
neuraminidase, was isolated from the mixed yield by
passage with antisera suppressive to viral·surface
antigens H3, H0, Neq1 and N1. A/PR/8/34 (HON1) did
not donate surface antigens to X-38 but presumably
provided unidentified genes (not shown) associated
with high virus yield in the chick embryo. X-37 was
derived in the same experiment as a virus serotypically
like A/Eng/42/72 and with high yield characteristics.

Table 1.   Characterization of vaccines

Vaccine	Activity		Immunogenic units*	
	Hemagglutinin	Neuraminidase	Hemagglutinin	Neuraminidase
X-37 (H3N2)	798**	12.5***	1280	640
X-38 (Heql N2)	643	9.1	640	640

*   per ml. of vaccine - antigenic extinction titrations in rabbits

** chick cell agglutinating units/ml.

*** n moles/ml/min. of N-acetyl neuraminic acid released from fetuin substrate

Table 2.   Serologic response to H3N2 and Heq1N2 vaccines

Vaccine	No. of Subjects	Homotypic HI response		NI response	
		No.	Mean	No.	Mean[4]
None	29	0	0	0	0
H3N2	36	26(72%)[1]	4.3X	9(25%)[3]	2X
Heq1N2	39	30(77%)[2]	2.6X	27(69%)[3]	4X

[1] vs. H3Neq1 virus
[2] vs. Heq1Neq1 virus
[3] vs. HOpr8N2 England virus
[4] mean fold increase in NI antibody

Table 3. Heterotypic HI response to Heq1N2 vaccine (induction of anti-H3 antibody)

Vaccine	No. of subjects	Homotypic HI response		Heterotypic HI response	
		No.	Mean	No.	Mean
None	29	0	0	0	0
H3N2	36	26 (72%)	4.3X	0	0
Heq1N2	39	30 (77%)	2.6X	22 (56%)	2.7X

VACCINE RESPONSE
NI TESTS WITH DIFFERENT N2 TEST ANTIGENS
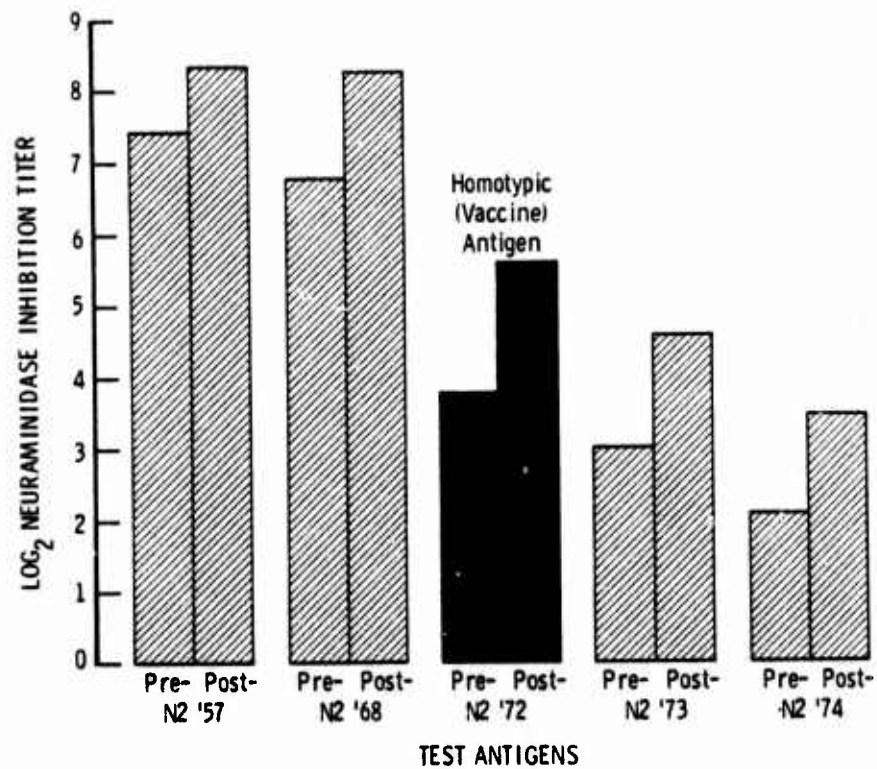
Figure 2.   Magnitude of anti-N2 antibody increase in
subjects immunized with X-37 (H3N2) (same population
as in Figure 2).   Maximum fold increases in titer are
demonstrated with the antigen homotypic to immunization
(N2'72).   However, absolute increase is greater to
earlier N2 antigens, illustrating "original antigenic
sin" or heterospecific anamnestic response.   (see text
discussion)

On the basis of an earlier study in human subjects
(1) it was anticipated that the administration of in-
fluenza viral neuraminidase in an antigenically hybrid
virus would prove immunogenic for antineuraminidase
antibody. Two unexpected findings in the present
comparison of an experimental vaccine of conventional
bispecific (H3N2) composition with a new antigenic
hybrid (Heq1N2) containing equal amounts of N2 antigens
were: 1) the superior potency of the hybrid vaccine
in engendering antineuraminidase response and 2) in-
duction of heterospecific (anti-H3) antibody by the
presumably neutral or irrelevant Heq1 hemagglutinin
of the recombinant hybrid vaccine strain.

In the earlier study, comparison of antineura-
minidase antibody responses with those with a vaccine
of conventional composition had not been made and all
subjects had been sero-negative for anti-H3 HI anti-
body prior to immunization, and pre-immunization anti-
NA antibody either was not demonstrable or was present
only in low concentration. Thus, lack of a contempor-
aneously administered, matched conventional vaccine
and the utilization of a relatively unprimed population,
probably precluded demonstration either of the hybrid
vaccine's superiority or heterotypic response in the
earlier study (1). It is notable that in studies of
another Heq1-containing hybrid vaccine, X-42, (Meiklejohn
and Eickhoff, personal communication) HI cross-reactions
detected with test virus bearing the N2 neuraminidase
(H3N2) were virtually eliminated when a hemagglutinin-
monospecific test antigen was employed. Furthermore,
HI heterotypic responses were absent in children immu-
nized with the same vaccine (X-42), probably in relation
to the lesser priming of that population with the H3
antigen (Ogra, et al., personal communication).

Of critical importance to the interpretation of
protection studies with antigenic hybrids containing
the Heq1 HA, are the preliminary data suggesting that
the heterotypic antibody induced is non-neutralizing
to H3-containing potential challenge viruses. Thus,
in accord with the assumptions on which the strategy
of immunization is based, any protection induced can

be interpreted as probably the consequence of increase
in antineuraminidase antibody alone. That is, signifi-
cant increase in neutralizing antibody might reduce
the probability of successful challenge infection
essential for the definitive immunizing step required
in the immunization strategem of neuraminidase-specific
vaccination. Therefore, it is encouraging that pre-
liminary evidence indicates that the heterotypic anti-
body that is formed is non-neutralizing. Efforts to
select a variant of Heq1 non-cross-reactive with H3
antigen have been frustrated thus far by the absence
of plaque neutralization of Heq1Neq1 virus by anti-H3
antiserum. This approach is reasonable in view of
evidence that influenza viral hemagglutinin contains
at least two antigenic determinants that are either
strain specific (homotypic) or cross-reactive (hetero-
typic), that are subject to independent variation
(2, 3). Indeed, elimination of cross-reactivity of
an H3 strain with Heq2 has been obtained by selective
passage of H3N2 virus with Heq2 antiserum, (Schulman,
J. L., unpublished data) although in that instance the
selected virus was still heterotypically immunogenic.
Complete elimination of hemagglutinin-mediated cross-
reactivity obviously can be achieved by the use of
isolated purified viral neuraminidase as antigen, but
studies of the immunogenicity of such preparations in
man are only in their inception. In the meantime,
the continued exploratory use of antigenic hybrids
as neuraminidase-specific immunogens should provide
useful information.

On the basis of previous study of age related
influenza viral neuraminidase antibody patterns of
man (4), and recognition of distinct antigenic neura-
minidase variants, even within the same viral subtype
(5), and recall of 1964 neuraminidase antibodies by
1968 influenza A virus immunization (6), it was not
surprising to find heterotypic anamnestic response to
the introduction of neuraminidase antigen. Response
in terms of number of subjects experiencing increase
in neuraminidase antibody arbitrarily defined as
significant was best demonstrated with homotypic
N2/72 antigen, but the amount of antibody formed was

greater to N2 antigens of earlier prevalence - those
with whom the population had had their initial ex-
perience. These observations reemphasize the question
already raised on the basis of heterotypic anamnestic
response to the hemagglutinin antigen of the wisdom
of vaccine strain changes in response to every minor
antigenic variation of the virus.

The immunogenic superiority of N2 antigen when
administered in an antigenically hybrid virus containing
hemagglutinin irrelevant to human experience was
unexpected. It must be emphasized that X-37 and X-38
vaccines were matched vaccines in every way. They were
produced simultaneously by the same manufacturer by
the same process and were standardized on the basis
of antigenic extinction titrations to equivalent immuno-
genicity in experimental animals. Furthermore, the
animal studies furnish direct evidence that when given
at equal dosage the two vaccines induced comparable
levels of NI antibody in primary immunization. Although
not necessarily related to immunogenicity, the neura-
minidase activity of the vaccines was comparable, with
the antigenic hybrid, X-38, actually having less activity
than the bispecific X-37 vaccine that is representative
of present conventional vaccines. These observations
are reinforced by those from study of the comparably
matched vaccines, X-41 and X-42 (Ogra, et al., personal
communication), in which the antigenic hybrid, X-42,
also induced greater frequency and magnitude of response
to the neuraminidase antigen.

Thus, explanation for the phenomenon must be
sought in differing immunologic response to presentation
of the neuraminidase antigen in association with dif-
ferent hemagglutinin antigens. In subjects primed with
H3 antigen, as was the case in the present study, the
processing of vaccine viral particles is probably
different. On the basis of immunologic memory, H3N2
viral particles may be preferentially directed to H3
memory cells (presumably B lymphocytes [3]), compared
to HeqlN2 particles for which the subjects are primed
principally with respect to N2. Although the mechanisms
and details of such antigen processing can only be

speculated upon, it is not unreasonable that the presence of a memory system for H3 might competitively reduce the opportunity for N2 to unite definitively with B or T cell receptors for the induction of anti-N2 response, when the H3 and N2 antigens are borne by the same particle.

In contrast, the absence in human subjects of an expanded population of memory cells for the alien antigen Heql might result in competition in which N2 antigen is preferentially absorbed by N2 memory cells in subjects given HeqlN2 vaccine, while response to the associated Heql antigen that reaches antibody forming cells is primary. This formulation ignores the demonstrated cross-reactivity of Heql and H3 antigens, but it can be assumed that the affinity of Heql for H3 memory cells would be less than in homotypic response. In fact, B cell-specific adoptive immunization experiments with purified hemagglutinin antigen of cross-reactive influenza viruses of other subtypes (HO and Hl) confirm a greater magnitude of homotypic than heterotypic response when primed animals are initially stimulated with the cross-reactive antigen (7).

Studies of cell mediated immunity in influenza are just beginning, and it is clear that they must take cognizance of possible differences in the processing of hemagglutinin and neuraminidase antigens as well as differing modulation of the immune response dependent upon varying backgrounds of immunizing experience and relatedness of variants of either antigen. All these factors muse be considered as experiments with neuraminidase-specific immunization are continued, and especially as this procedure is employed as a prelude to administration of live virus vaccines.
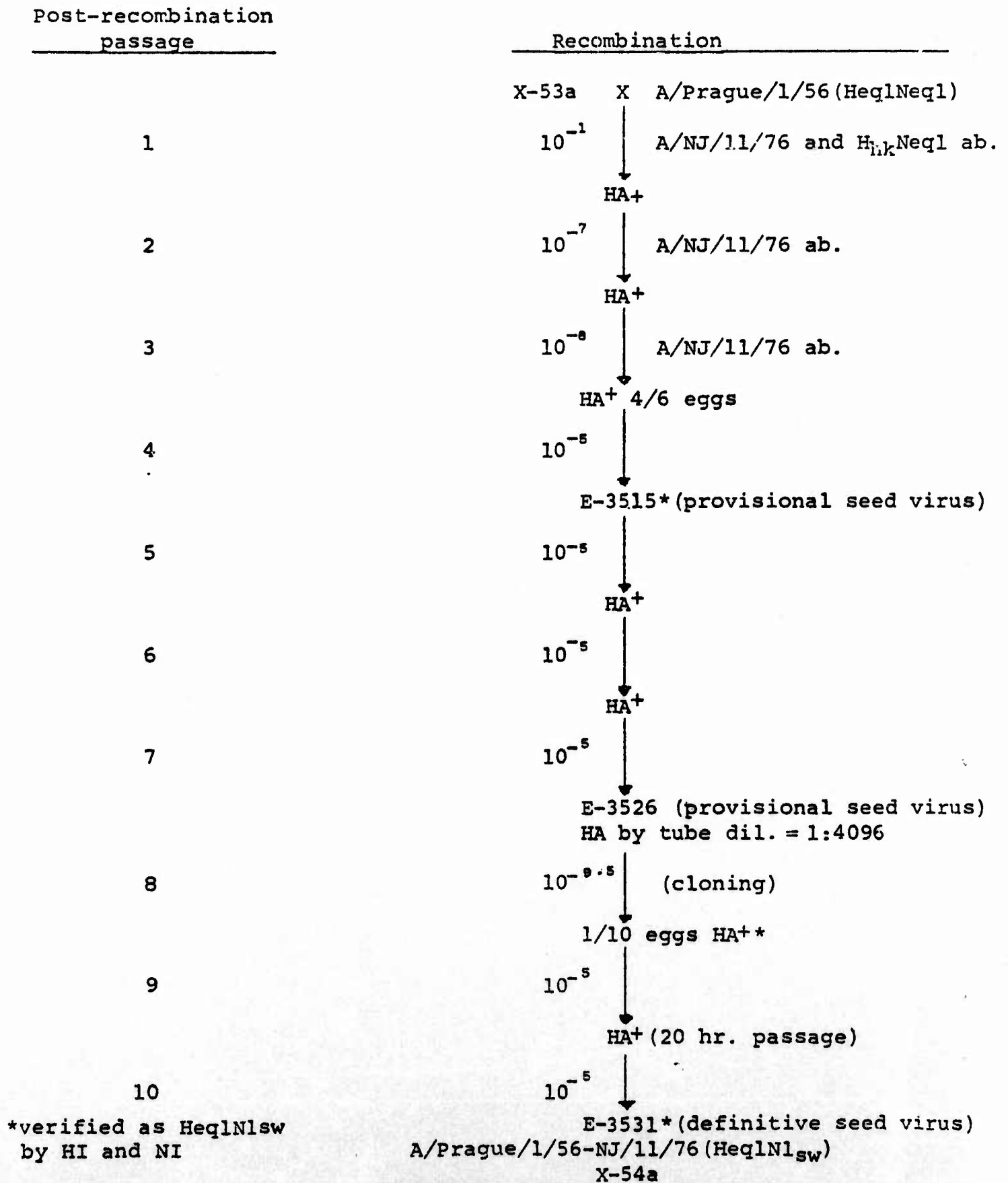
Preparation of an antigenic hybrid for use in neuraminidase-specific immunization against swine influenza

With the appearance of any new influenza virus variant it has been our custom to prepare an antigenic hybrid that incorporates the Heql hemagglutinin and the neuraminidase of the new strain. Such recombination

assumes special significance in the case of the
Ft. Dix A/sw/NJ/76 virus to which younger members
of the population lack antibody.   Therefore an
unprecedented opportunity is provided for the
primary antigenic stimulation of humans by mono-
specific immunization with a neuraminidase to the
swine virus.   Recombination as indicated in Figure 3
was carried out employing X-53a, the high yielding
A/sw/NJ/11/76 recombinant used in vaccine production
and A/Prague/1/56 (Heq1Neq1) the usual hemagglutinin
donor.   Derivation of the new hybrid X-54a is shown
in Figure 3.   This virus is now in experimental
production by Lederle Laboratories for use in pro-
jected field trials that may receive NIAID sponsorship.

Figure 3.

Derivation of X-54a antigenically hybrid recombinant
$[\text{Heq1N1}_{sw(NJ)}]$

Post-recombination passage	Recombination
	X-53a X A/Prague/1/56 (Heq1Neq1)
1	10^{-1} | A/NJ/11/76 and H_{lik}Neq1 ab.
	HA+
2	10^{-7} | A/NJ/11/76 ab.
	HA+
3	10^{-8} | A/NJ/11/76 ab.
	HA+ 4/6 eggs
4	10^{-5}
	E-3515*(provisional seed virus)
5	10^{-5}
	HA+
6	10^{-5}
	HA+
7	10^{-5}
	E-3526 (provisional seed virus) HA by tube dil. = 1:4096
8	$10^{-9.5}$ | (cloning)
	1/10 eggs HA+*
9	10^{-5}
	HA+ (20 hr. passage)
10	10^{-5}
*verified as Heq1N1sw by HI and NI	E-3531*(definitive seed virus) A/Prague/1/56-NJ/11/76 (Heq1N1$_{sw}$) X-54a

Neuraminidase vaccine of isolated viral protein
(with Douglas and Cerini, Lederle Laboratory)

Preparation of the vaccine

Neuraminidase can be purified from influenza
virus or vaccine by adsorption and elution from an
affinity column bearing an inhibitor to neuraminidase,
aminophenol oxamic acid, as originally devised by
Cuatrecasas and Illiano (1971) (8) and subsequently
modified for purification of the viral neuraminidase
([9] and earlier progress reports). There was an
average increase of 10.6 fold in specific activity
when X-38 vaccine was the starting preparation.

The availability of the purified neuraminidase
from influenza vaccine in milligram quantities permits
the testing of the neuraminidase-specific infection-
permissive vaccine using the neuraminidase as the
immunizing agent. The concept of the neuraminidase-
specific vaccine has been developed by E. D. Kilbourne
and associates, 1972 (10), and Couch and associates,
1974 (1). Use of the purified neuraminidase free
of hemagglutinin would eliminate the problem of cross-
reactivity of the "irrelevant" hemagglutinin in the
recombinants originally proposed as the source for
a neuraminidase-specific vaccine. An additional
advantage of the use of the purified neuraminidase
as the vaccine would be the probable lowered reacto-
genicity of such a preparation. Subunit vaccines
prepared by disruption of viral particles with sodium
deoxycholate have been shown to be non-pyrogenic (11).

The feasibility of preparing such a vaccine
under sterile conditions for clinical trials was
tested in our laboratory. The starting preparation
was a commercial vaccine of MRC-11, a high yielding
recombinant of "London flu" virus. An affinity
column was prepared by the usual procedure under
sterile conditions (8, 9). All buffers and glassware
were sterilized. The purification technique was that
generally employed for the preparation of neuraminidase
from virus or vaccine lots. The one major change
required for the preparation of vaccine suitable for

administration to human subjects was the substitution
of Tween 80 (considered an "injectable" by the FDA)
for that of Triton X-100. Both compounds are non-
ionic detergents and substitution of the Tween 80 for
Triton X-100 was not a major barrier. Triton X-100
is used in the purification of neuraminidase as a
stabilizing agent rather than as a disrupting agent.
Generally Triton X-100 is added at a level of 0.1 - 0.2%
to all buffers employed in adsorption and elution and
to a level of 10% to the viral preparation after dis-
ruption with 1% SDS. If these same conditions were
maintained but Tween 80 substituted for Triton X-100,
the recovery of neuraminidase activity from the
affinity column was only 1.7%. If the level of Tween 80
was elevated to 0.5% in the buffers and to 20% after
the SDS disruption step, then the recovery of neura-
minidase from the affinity column was 25%. Reduction
of the anionic detergent SDS to a level of 0.5% (from
1%), resulted in elution of the enzyme from the column
with yields of 32 - 49%. The final preparation of
neuraminidase employed for vaccine in clinical trials
represented a yield of 32% and had an increase in
specific activity of 3.7 fold. Additional denaturation
occurred with the "packaging" of the neuraminidase.
The neuraminidase vaccine in the vials had a specific
activity only two fold higher than the starting vaccine
preparation: a stabilizing agent may be necessary for
preservation of enzyme activity. This low increase
in specific activity was apparently due to a substantial
amount of contamination with nucleoprotein as visualized
on polyacrylamide gels. A total of five vaccine batches
were prepared. Two batches had a substantial quantity
of hemagglutinin remaining as seen on polyacrylamide
gels; however, these preparations were not used in the
vaccine trial. This contamination was apparently due
to the use of excessively mild conditions for disruption
of the viral particles with SDS; these preparations had
been disrupted with SDS at 4°, cleaner preparations were
obtained when the disruption with SDS was performed at
room temperature.

An additional complication in the procedure was
the presence of 0.25% Tween 80 in the starting vaccine
preparation. Since the nonionic detergent probably
has a protective effect, countering the disruption of
the viral particles with SDS and resulting in con-
tamination of other viral proteins, future neuramini-
dade vaccines will be prepared after centrifuging
the viral particles from the vaccine solution to
eliminate most of the Tween 80 before the SDS dis-
ruption step.

An additional concern had been the contamination
of the neuraminidase with amino-phenol oxamic acid
from the affinity column. A certain amount of "bleed"
does occur with the first few cycles of a new column.
Therefore a series of three trial runs was conducted
before the actual vaccine batches were adsorbed and
eluted from the column. The minimal level of detection
was 4 nanograms/ml. The first of the five vaccine
batches had a level of 40 nanograms/ml. However, the
final preparation used in the clinical trials contained
no detectable amino-phenol oxamic acid.

An additional benefit of the chromatographic
preparation of the neuraminidase was the substantial
lowering of endotoxin contamination. Whereas the
original vaccine preparation required a dilution 1:200
to reach an endpoint in the limulus assay, the purified
neuraminidase had an endpoint of 1:10.

Preliminary studies of antigenicity of neuraminidase vaccine

At the dose level of 0.1 ml., adjusted to the
volume of a normal vaccine dose on the basis of neura-
minidase activity, three out of the four subjects
responded with 8 to 25 fold increases in antibody
level as measured by neuraminidase inhibition with
an average increase of 17 fold (see Table 4). However,
three out of the four subjects responded with an in-
crease in antibody to hemagglutinin, one at a 2 fold
level, two at a four fold increase, representing an
average increase of 3 fold.

Table 4.   Serologic response to purified neuraminidase

Vaccine dose	No. of subjects	HI response[1]		NI response[a]	
		No.	Mean fold increase	No.	Mean fold increase
0.1 ml	4	3	3	3	17
0.5-2.0 ml	6	6	19	6	13

[1] versus H3 (Pt. Chalmers)/Neq1 recombinant
[a] versus Heq1/N2 (Pt. Chalmers) recombinant

At dose levels of five to 20 fold higher levels of the neuraminidase vaccine, the six subjects had 4 to 32 fold increases in neuraminidase inhibiting antibodies, representing an average increase of 13 fold. At these high doses, the hemagglutinin contamination became quite obvious with antibody responses measured as hemagglutination inhibition ranging from two to 64 fold, with an average increase of 19 fold.

Thus it can be seen that good immunologic response to the neuraminidase was achieved at 0.1 ml., a dose equivalent to the neuraminidase activity in the original MRC-11 vaccine. Therefore, the purified neuraminidase possesses good immunogenicity and it is apparently valid to adjust the dose to equivalence with the neuraminidase activity of the intact virus vaccine. Higher doses of the neuraminidase vaccine did not result in any appreciable increase in antibodies to neuraminidase but did produce a significant antibody response to the hemagglutinin which apparently contaminated this preparation. Modifications can be introduced at several points to eliminate such contamination in the future.

More severe conditions for disruption of the viral particles at the point of addition of SDS may be of value. These conditions would include elevation of the SDS level to 1% and the elimination of the 0.25% Tween 80 from the vaccine preparation before SDS disruption.

Additionally, tailor-made recombinants may be used to eliminate contamination with hemagglutinin. These would include the use of hemagglutinins in recombinants which are SDS-sensitive, such as the hemagglutinin from WS or certain H2N2 strains (Webster and Laver, 1966) and/or the use of "irrelevant" hemagglutinin which would have low cross-reactivity if not completely removed.

In summary, these preliminary results indicate that the purified neuraminidase possesses good immuno- genicity and the feasibility of the preparation of such a vaccine has been demonstrated. However, future vaccine preparations will require additional testing to insure that all contaminating hemagglutinin has been removed.

Chromatographic isolation of the viral polypeptides

Modifications of the chromatographic system have included the use of Sepharose 6B-CL for the first stage of separation of the HA-NP fraction. This gel filtration material is considerably more rigid due to the introduction of cross-linking groups in the gel. Since the gel material is more rigid, higher concentrations of protein can be applied permitting the separation of very large quantities of the purified proteins. The sample is applied in 20 ml. quantities of very high viscosity. Although the Bio Gel A-5m permits finer resolution, it is a very soft gel which tends to compact and shut off the flow rate with viscous samples. The column technique now includes a first stage separation of the very viscous total viral proteins on Sepharose 6B-CL followed by concen- tration and reapplication of the HA-NP fraction to Bio Gel A-5m permitting fine resolution of this fraction and improving separation of HA-NP from P and M. The HA-NP fraction is then reduced and reapplied to the Bio Gel A-5m solumn to separate the HA_1 from HA_2.

The SDS can now be removed from the protein using a new technique which we have developed which involves the use of an anion exchange gel with 6 M urea. The substitution of anion exchange gel (QAE Sephadex) for DEAE cellulose greatly improves the protein yield from the column. The 6 M urea must be included to insure quantitative recovery. Whereas the DEAE cellulose permitted recovery of 40 - 80% of the viral protein with removal of detergent, the QAE Sephadex permits recovery of 80 - 100% of the total protein. This change in technique permits a very considerable improvement in yield of protein and allows a large savings in material.

Formation of immunogenic soluble hemagglutinin --

Relationship of the substructure of hemagglutinin to
immunogenicity

It has become apparent that the immunogenicity
is related to the degree of substructure of the
hemagglutinin. With the use of a lower ratio of
SDS/protein for disruption of the viral particles,
a large proportion of the hemagglutinin migrates as
a 150,000 component on SDS gel filtration rather
than the 75,000 unit -- suggesting a dimeric asso-
ciation of the hemagglutinin. Use of the "dimeric"
hemagglutinin as an immunogen for rabbits has resulted
in the development of hemagglutination inhibiting (HI)
antibodies, whereas previously only a very low HI
response could be obtained with HA_1, HA_2 or HA-NP.

The immunogenicity of HA_1 and HA_2 as separated
by SDS gel filtration has been shown to be very low
(Erickson and Kilbourne, unpublished results). The
HA_1 and HA_2 polypeptides were chromatographed to
remove SDS and "renatured" according to Weber and
Kuter (1971) (12). The HA-NP fraction was similarly
treated. Quantities of 100 micrograms were used for
initial immunization and the subsequent booster dose.
The HI response was 1:40 to 1:80. A similar quantity
of "dimeric" hemagglutinin under the same conditions
resulted in an HI of 1280 under similar conditions,
either with or without prior removal of the SDS.

From the chromatographic elution of the hemag-
glutinin polypeptide(s) on SDS gel filtration, it is
apparent that there are two states of SDS solubili-
zation of the hemagglutinin spike: (1) occurs with
release of the immunologically active state probably
as a dimer aggregate and (2) additional SDS converts
the HA dimer to the monomeric form which is anti-
genically and biologically inactive. The addition
of a reducing agent to the monomeric hemagglutinin
converts the glycoprotein to HA_1 plus HA_2. The
various proposed states of existence of the hemag-
glutinin polypeptide(s) in the presence of SDS are

diagrammed in Figure 4. These findings help to
explain the anomaly of SDS "resistant" and "sensitive"
hemagglutinins and the corresponding association of
activity with these preparations (11). Those hemag-
glutinins which appear "resistant" to SDS probably
bind HA poorly and are in the dimeric form, those
hemagglutinins which are "sensitive" would readily
bind SDS and be converted to the monomeric form which
is no longer immunogenic. Thus careful control of
detergent level can yield hemagglutinin in the dimeric
form which can be acetone or ethanol precipitated from
the SDS containing solution and which possesses
biologic activity. The detergent/protein ratio is
important in determining biologic and immunologic
activity.

Evidence from hemolysis of red blood cells would
suggest that at least twice as much detergent is
present in a similar volume for the monomeric as for
the dimeric form. However, the detergent to protein ratio
has not been calculated on a molar basis and must be
determined for an accurate understanding of detergent/
protein interaction.

PROPOSED RELATIONSHIP OF SUBSTRUCTURE OF
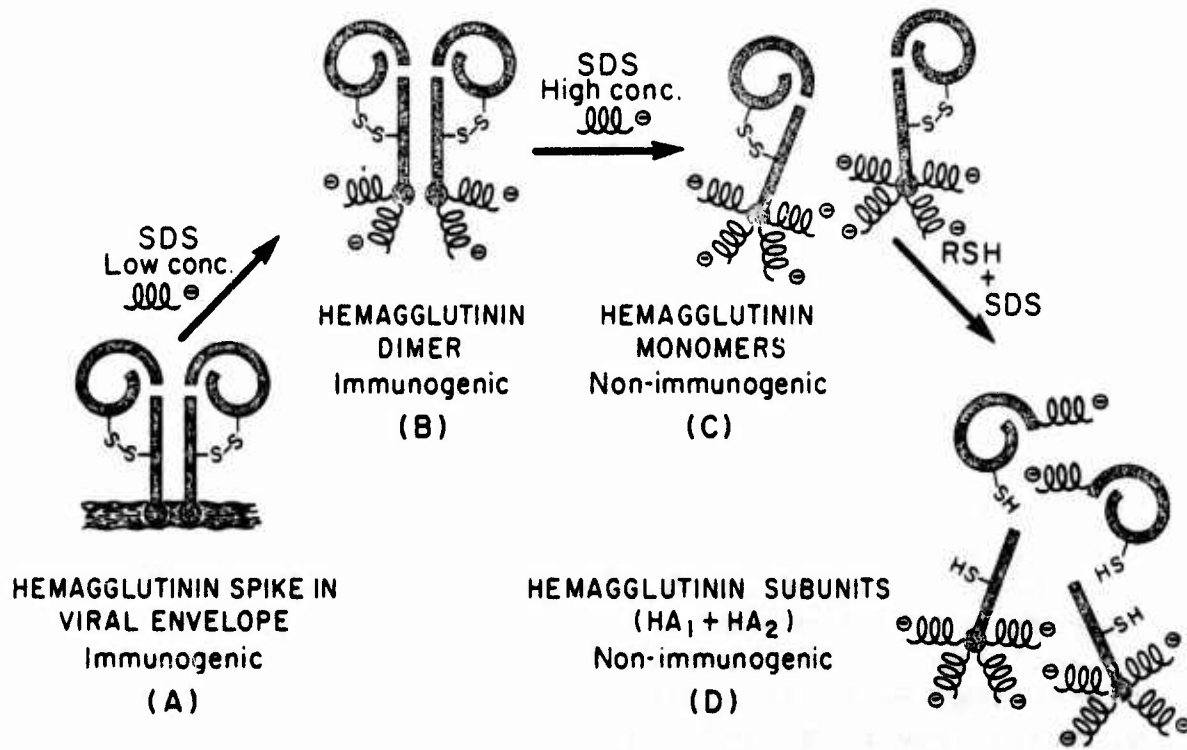HEMAGGLUTININ TO IMMUNOGENICITY

Figure 4.

Pyrogenicity experiments (with Dr. Atkins)

The accompanying high fevers frequently occurring in persons immunized with influenza vaccine continue to be a problem even with the highly purified virus preparations obtained through gradient separation of the virus. This undesirable side effect leads to a low acceptance rate of the vaccine even though the vaccine can be highly effective.

In collaboration with Dr. Atkins we are seeking the pyrogen producing component(s) of the influenza virion. With the identification of this factor, the component could be eliminated and vaccines free of pyrogen inducer could be produced. With the SDS gel filtration technique of separation of the viral components, yielding all the fractions of the virus, it is possible to test each component independently for its pyrogenic effect. It has been previously shown that the release of endogenous pyrogen in rabbits is dependent on the presence of a biologically active hemagglutinin. The pyrogen inducer, however, can be inactivated by UV irradiation even though the hemagglutinin remains active. Thus the pyrogenic factor is not the hemagglutinin but requires its activity to cause the induction of endogenous pyrogen. Subunit vaccines are known to be free of pyrogenic factor and thus far it has not been possible to recombine fractions from the subunit vaccines to reactivate the pyrogenic factor.

Sterile virus preparations free of endotoxin have been produced in the laboratory to examine the pyrogenic effect of intact virus in rabbits. Dr. Atkins has been examining two distinctly different types of influenza viruses -- Aichi and a swine flu recombinant (X-53a). The swine flu recombinant did not produce any pyrogenic response even at undiluted concentrations. The Aichi strain did produce a fever in rabbits, apparently not of the endotoxin type.

Both preparations were quite dilute, 128 and 256 HAU/ml,
respectively, and new preparations of higher concen-
trations are being processed.   These preparations
were purified on sucrose gradients, dialyzed versus
PBS and subsequently tested in rabbits.   It is possible
that the factor inducing pyrogenicity is unstable
and/or dialyzable with exhaustive dialysis.

In addition, Dr. Atkins has been testing several
vaccine preparations from the Bureau of Biologics,
including swine flu vaccines prepared by four pharma-
ceutical concerns.   One of the swine flu vaccines was
shown to be highly reactogenic in humans although
relatively free of endotoxin.   Currently these
preparations are being studied with the aim of
determining the degree of correlation of pyrogenicity
in rabbits with reactogenicity in humans.

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