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RAPID DETECTION OF ENVELOPED VIRUSES

ANNUAL REPORT

DORIS J. BUCHER, Ph.D.

November 27, 1986

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<b>19. ABSTRACT (Continue on reverse if necessary and identify by block number)</b> <p>We have continued to enhance the sensitivity of our type A influenza virus detection system utilizing the monoclonal antibodies to M-protein. Based on the results of variation of parameters of our capture assay system developed at the start of the influenza season this past winter, we used a combination of purified monoclonal antibodies, 2B-B10-C12 and 1G8-A11, to coat the microtiter plates for use as capture antibodies. More recent work with combinations of monoclonal antibodies (some lines developed in the past year) and epitope analysis (see below) suggest a better combination is 2B-B10-C12 (or G9) and 611-G10-D3; this combination will be used as the capture phase for analysis of specimens from the 1986-87 winter influenza season.</p> <p>Analysis of clinical specimens showed a distinctly higher reactivity with a collection of H1N1 positive specimens obtained from Dr. Meikeljohn's</p>						
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laboratory as compared with negative controls. Earlier results with our own collection of H3N2 specimens (Annual Report, 11/27/85) also showed a higher degree of reactivity as compared with controls. Results with both collections support the validity of use of M-protein capture monoclonal antibodies for detection of type A influenza viruses.

Good ELISA reactivity was seen following amplification of 1984-85 H3N2 positive specimens in eggs suggesting the use of the M-capture system for typing influenza viruses. Considering the vagaries of the hemagglutination inhibition test, the M-capture ELISA system may be highly useful in typing influenza isolates. However, H3N2 specimens from the 1985-86 influenza season amplified in MDCK cells have dramatically lower reactivity than observed for H3N2 isolates made in the 1984-85 winter. Further investigation is necessary to determine if the epitope of M-protein reactive with the monoclonal antibodies has mutated (or is the result of a reassortment event) in the H3N2 influenza viruses circulating in 1986.

Several new hybridoma lines secreting monoclonal antibodies to M-protein have been developed and cloned. We are currently purifying antibodies from ascites fluids for these lines for analysis of epitopes and performance in the capture assay system. One line, 611-G10-D3, secreting an IgM reactive to M-protein, has been found to enhance the sensitivity of the capture system in combination with 2B-B10-C12 (or G9). Several lines also perform well in immunofluorescence assay, a methodology which may also be used for virus detection.

→ Combinations of purified monoclonal antibodies to M-protein used as capture antibodies for type A influenza viruses were superior to the use of a single monoclonal antibody for virus capture. The combination which produced the most sensitive level of virus detection was 2B-B10-C12 and 611-G10-D3. 1G11-D11 and 2B-B10-C12 also provided sensitive detection for X-53a (H1N1) virus although this combination did not provide sensitive detection of 15-2 (H3N2) virus.

→ These results correlate with our findings on epitope analysis of the monoclonal antibodies to M-protein. Results from competitive inhibition of alkaline phosphatase conjugated monoclonal antibodies with unconjugated monoclonal antibodies suggest that 2B-B10-C12 (G9 and A5) and 1G8-A11 are directed against one epitope, 611-G10-D3 is directed against a second epitope and 1G11-D11 interacts with a third epitope. → Therefore, it would be predicted that combinations of monoclonal antibodies recognizing different epitopes would provide maximal sensitivity.

A Western blot technique utilizing enzyme immunoassay visualization was developed. All monoclonal antibodies previously found to react with M-protein by microtiter ELISA assay also reacted with M-protein by the Western blot technique.

Our collaborative work with Dr. Judd at S.R.I. on the production of immunoreactive peptide segments of M-protein is important to this project from at least two aspects. Important applications for these findings are: (1) Synthetic peptide segments of M-protein can be used in ELISA assays as adsorbents to determine the precise epitopes seen by monoclonal antibodies to M-protein. (2) Synthetic peptides of regions of M-protein may serve as adsorbent antigen in place of M-protein in viral detection systems based on competitive inhibition assay.

## SUMMARY

We have continued to enhance the sensitivity of our type A influenza virus detection system utilizing the monoclonal antibodies to M-protein. Based on the results of variation of parameters of our capture assay system developed at the start of the influenza season this past winter, we used a combination of purified monoclonal antibodies, 2B-B10-C12 and 1G8-A11, to coat the microtiter plates for use as capture antibodies. More recent work with combinations of monoclonal antibodies (some lines developed in the past year) and epitope analysis (see below) suggest a better combination is 2B-B10-C12 (or G-9) and 611-G10-D3; this combination will be used as the capture phase for analysis of specimens from the 1986-1987 winter influenza season.

Analysis of clinical specimens showed a distinctly higher reactivity with a collection of H1N1 positive specimens obtained from Dr. Meikeljohn's laboratory as compared with negative controls. Earlier results with our own collection of H3N2 specimens (Annual Report, 11/27/85) also showed a higher degree of reactivity as compared with controls. Results with both collections support the validity of use of M-protein capture monoclonal antibodies for detection of type A influenza viruses.

Good ELISA reactivity was seen following amplification of 1984-85 H3N2 positive specimens in eggs suggesting the use of the M-capture system for typing influenza viruses. Considering the vagaries of the hemagglutination inhibition test, the M-capture ELISA system may be highly useful in typing influenza isolates. However, H3N2 specimens from the 1985-86 influenza season amplified in MDCK cells have dramatically lower reactivity than observed for H3N2 isolates made in the 1984-85 winter. Further investigation is necessary to determine if the epitope of M-protein reactive with the monoclonal antibodies has mutated (or is the result of a reassortment event) in the H3N2 influenza viruses circulating in 1986.

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Combinations of purified monoclonal antibodies to M-protein used as capture antibodies for type A influenza viruses were superior to the use of a single monoclonal antibody for virus capture. The combination which produced the most sensitive level of virus detection was 2B-B10-C12 and 611-G10-D3. 1G11-D11 and 2B-B10-C12 also provided sensitive detection for X-53a (H1N1) virus although this combination did not provide sensitive detection of 15-2 (H3N2) virus.

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I. Influenza Surveillance - 1985 - 1986 Winter (11/12/85 - 3/18/86).

In the Winter of 1985-86 we expanded our surveillance activities of individuals with respiratory tract infections considerably over the previous influenza season. In addition to our weekly sampling at Mount Sinai to obtain nasal washings from children, we enlisted the cooperation of the Director of Employee Health Services, Dr. Donald Smith, and obtained 14 throat wash specimens from Mount Sinai employees. We also obtained several positive specimens and performed the serotyping of isolates for Dr. Ed Desmond of the Bronx VA Hospital. One set of five nasal wash specimens from Jersey Shore Medical Center was provided by a visiting fellow.

A total of 81 specimens were collected from the pediatric clinic which included 71 nasal wash specimens from children and 10 throat washings from adults. Three type A (H3N2) and two type B isolates were made from this population. One type A (H3N2) isolate was made from the 14 throat-wash specimens collected at Mount Sinai Employee Health Services. One type B isolate was made from the Jersey Shore Medical Center specimens. One type A (H3N2) and three type B's were serotyped from the Bronx VA isolates. Specimens from these various sources totaled 104; five type A (H3N2) and six type B isolates were identified.

We were also fortunate in gaining the cooperation of the Nassau County Medical Center Virology Laboratory, directed by Dr. Steve Lipson. We sampled possible flu specimens (throat and nasopharyngeal swabs) on a weekly basis from February 14 through March 14, 1986. We tested a total of 185 specimens (throat and nasopharyngeal swabs) on a weekly basis from February 14 through March 14, 1986. These specimens were analyzed by ELISA and placed into tissue culture (MDCK cells, 35 mm dishes) to verify Dr. Lipson's results. Pronounced CPE was associated with positive specimens (see figure 1). This group of specimens yielded 8 type A (H3N2) and 11 type B influenza virus isolates.

The specimens were collected by a variety of means. Specimens collected from children at Mount Sinai were nasal washes made with sterile saline. Adult specimens at Mount Sinai were saline 'gargles'. Specimens from the Bronx VA Hospital and Nassau County Medical Center were throat and/or nasopharyngeal swabs placed in viral transport media. Nasal washings would be expected to have the highest antigen concentration, lower amounts of antigen would be found in throat washings and swabs.

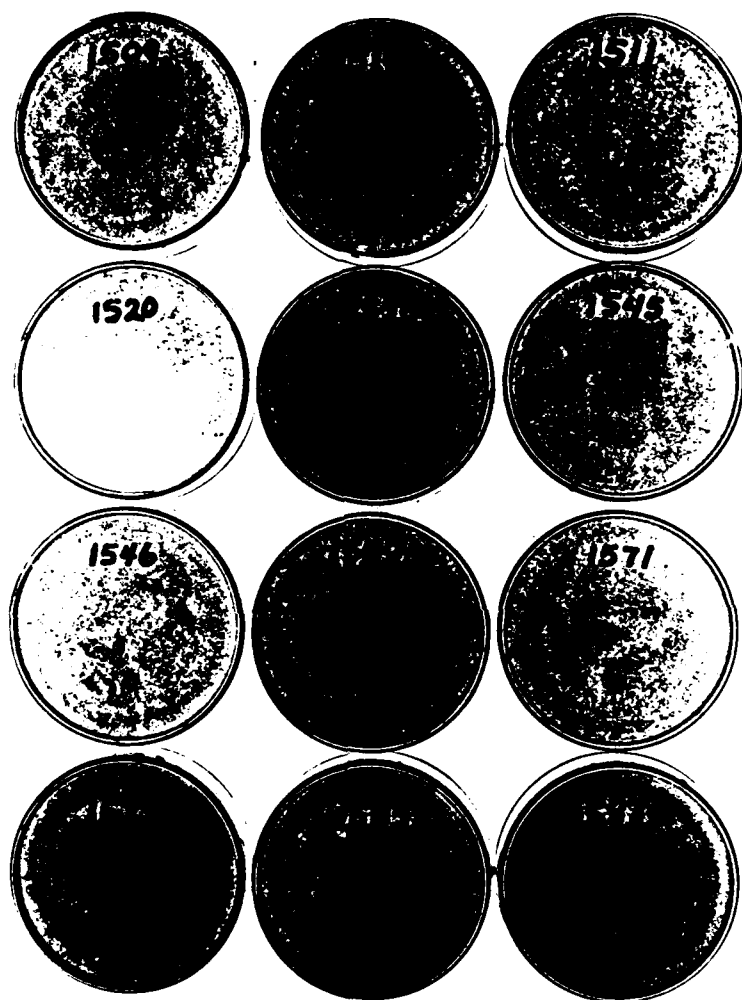


Figure 1. Tissue culture analysis of clinical specimens from Nassau County Medical Center. MDCK monolayers were exposed to 0.1 ml specimen (diluted 1:10), overlaid with media (no agar), incubated for three days and stained with crystal violet. Specimen 1520 was positive for type A influenza virus.

The influenza season in the winter of 1985-86 began much later than in the previous year. The first isolate was made from a specimen collected January 21, 1986, and the last isolate was made from a specimen collected February 27, 1986. This is in contrast to the previous winter when the first isolate was made from a specimen obtained December 10, 1984, and the last isolate was made one month later from a specimen collected on January 10, 1985.

Early isolates in this season were type A (H3N2) influenza virus with later isolates predominantly type B. In the previous season only type A (H3N2) isolates were made.

A copy of our report to Dr. Maurice Harmon of the CDC and a summary of isolates made in our laboratory at Mount Sinai is on pp. 4-5. Unfortunately, our number of isolates made at Mount Sinai was limited. Both type A and type B influenza virus cases were seen. Only three type A isolates were found among the nasal wash specimens. The Nassau County specimens provided us with a much larger number of cases but the level of antigen present would also be expected to be much lower in the throat and nasopharyngeal swab specimens.



OF THE CITY UNIVERSITY  
OF NEW YORK

# THE MOUNT SINAI MEDICAL CENTER

ONE GUSTAVE L. LEVY PLACE • NEW YORK, N.Y. 10029



Mount Sinai School of Medicine • The Mount Sinai Hospital

Department of Microbiology

March 27, 1986

Dr. Maurice Harmon  
Supervisor, Reference Lab  
Division of Viral Diseases  
Center for Infectious Diseases  
Centers for Disease Control  
Atlanta, GA 30333

Dear Maurice:

We've evidently seen the last of our flu cases for the season.  
For your records:

We monitored the pediatric clinic and in-patient population at Mt. Sinai on a weekly basis beginning Nov. 12 through Jan. 14 and collected a total of 37 specimens with no positives. From Jan. 21 through March 18 we collected an additional 44 specimens and found 5 positives. Type A (H3) isolates were made on 1/21/86, 2/4/86, and on 2/13/86. Type B isolates were made on 2/13/86 and 2/27/86.

Specimens were collected from employees attending the Mt. Sinai Health Service clinic from 2/6/86 through 2/11/86. Fourteen specimens were collected and one type A (H3) isolate was made on 2/11/86.

We also collected 5 specimens from children at the Jersey Shore Medical Center on 2/9/86 and obtained one type B isolate.

In addition we typed HA + isolates from specimens collected from adults at the Bronx VA by Dr. Ed Desmond. We typed a total of four isolates; one type A (H3) collected 1/29/86 and three type Bs collected on 1/29/86, 2/13/86 and 2/19/86.

The isolates have been amplified in MDCK cells. Please let me know if you would like any of them shipped to your lab.

Best regards,

Doris Bucher, Ph.D.  
Associate Professor of Microbiology

3/27/86

Specimen SummaryMt. Sinai pediatric clinic and in-patient population

<u>date</u>	<u># specimens</u>	<u>#positive</u>	
11/12/85	5	0	
11/19/85	3	0	
12/3/85	6(incl. 1 adult)	0	
12/10/85	3(incl. 1 adult)	0	
12/17/85	5	0	
12/26/85	5	0	
1/2/86	4	0	
1/9/86	3	0	
1/14/86	3	0	(37 specimens, 0 +)
-----			
1/21/86	6	1	type A (H3)
2/3/86	3(incl. 3 adults)	0	
2/4/86	7(incl. 2 adults)	1	type A (H3)-child
2/13/86	9(incl. 1 adult)	2	1 type A (H3)-child 1 type B-child
2/20/86	5(incl. 2 adults)	0	
2/25/86	3	0	
2/27/86	3	1	type B
3/6/86	5	0	
3/11/86	2	0	
3/18/86	1	0	(44 specimens, 5 +)
			total of 81 specimens

Mt. Sinai Employee Health Service (adults)

<u>date</u>	<u># specimens</u>	<u># positive</u>	
2/6/86	6	0	
2/7/86	3	0	
2/10/86	2	0	
2/11/86	3	1	type A (H3)

Jersey Shore Medical Center

<u>date</u>	<u># specimens</u>	<u># positive</u>	
2/9/86	5 (children)	1	type B

Bronx VA - HA+ specimens typed (all adults)

<u>date collected</u>	<u>typing</u>
1/29/86	1 type A (H3); 1 type B
2/13/86	1 type B
2/19/86	1 type B

## II. Parameters of ELISA test system

We continued to examine a number of different parameters in the ELISA assay to further enhance the sensitivity of our test system. We also examined the mechanism of detection of M-protein in this system in light of our earlier findings of selective adsorption of M-protein with the use of polystyrene beads (Bucher and coworkers, in press).

### A. The necessity for use of capture antibodies to detect M-protein

In our initial work on detection of influenza viruses through detection of M-protein using polystyrene beads we found that capture antibodies were unnecessary for adsorption of M-protein to the solid phase. Relatively harsh conditions of heat and detergent could be used to break open the virus, release M-protein and cause it to be adsorbed to the polystyrene beads (Bucher and coworkers, in press). In light of our earlier observations, we wanted to determine if the monoclonal antibodies were functioning as capture antibodies or was detection of M-protein occurring by selective adsorption of M-protein under the milder conditions employed in this assay.

Immulon 1 plates were left uncoated or coated with a combination of 2B-B10-C12 and 1G8-A11 purified monoclonal antibodies (2  $\mu$ g of each antibody in 200  $\mu$ l). A preparation of X-53a virus was assayed according to the usual protocol as described in the Annual Progress Report (11/27/85) (also see p12 of this report). The results leave no question that the monoclonal antibodies are serving a capture role; the use of a coating of monoclonal antibodies greatly enhanced virus detection under the conditions used in this assay (see Figure 2).

### B. Use of monoclonal antibodies as capture antibodies

We had observed earlier in test development that the use of monoclonal antibodies to M-protein as capture antibodies resulted in a more sensitive assay for virus than the use of purified polyclonal (rabbit) antibodies. Our system utilizes monoclonal antibodies to capture M-protein, rabbit antibodies to 'sandwich' the assay, and enzyme conjugated goat anti-rabbit antibodies for color detection. Since we had altered a number of parameters in test development, we decided to re-examine the use of polyclonal antibodies as capture antibodies and use the monoclonal antibodies as sandwich with enzyme-conjugated goat anti-mouse antibodies for detection. The results were dramatically in favor of the continued use of monoclonal antibodies as the capture phase (see Figure 3). Almost no detection was achieved when polyclonal antibodies were used as the capture phase. In addition, the background was substantially higher when rabbit antibodies were used as capture antibodies.

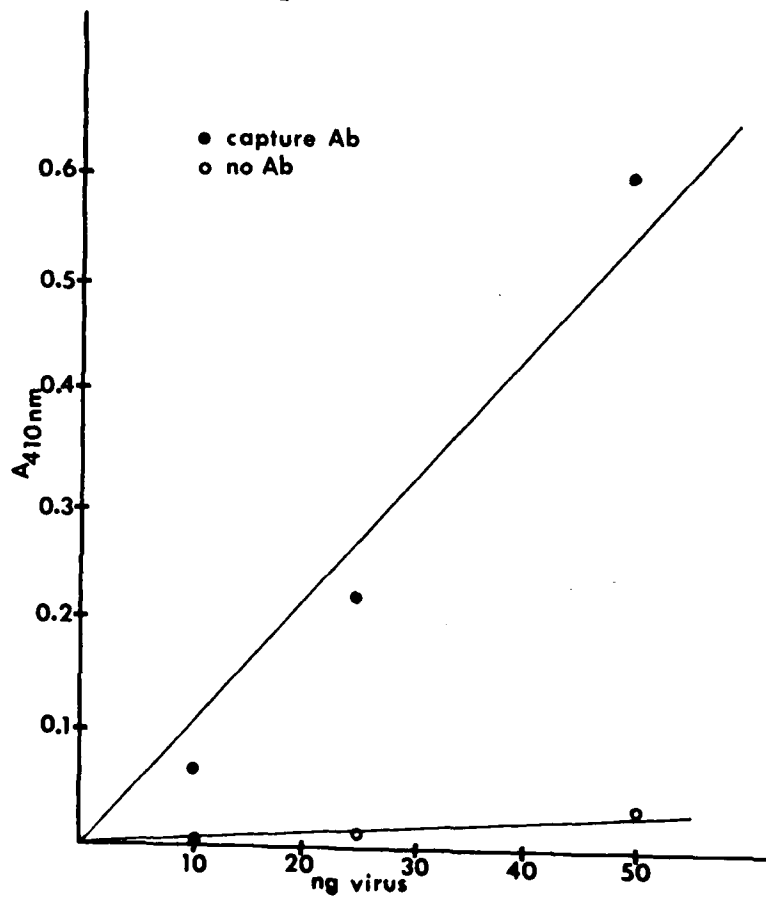


Figure 2. ELISA response to virus standard (X-53a) in the presence (●) or absence (○) of capture monoclonal antibody (2B-B10-C12 and 1G8-A11).

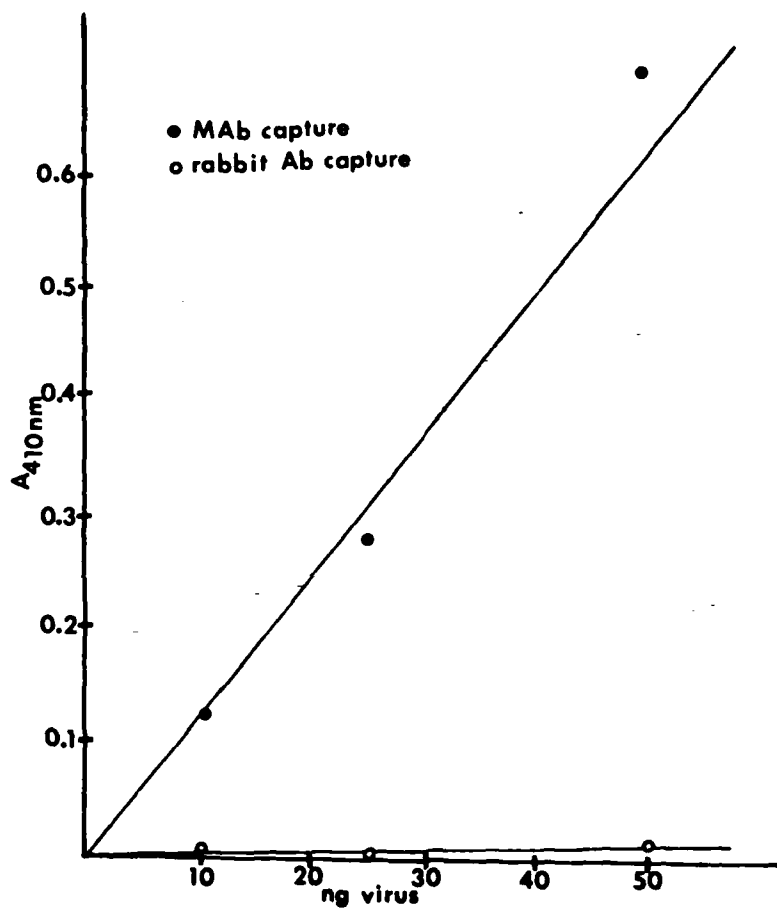


Figure 3. ELISA assay for standard virus (X-53a) in the presence of monoclonal antibody (●) or rabbit antibody (○) as the capturing antibody. Sandwich antibody is the opposite species; rabbit antibody in the first case (●) and monoclonal antibody (○) for the latter.

The almost total lack of detection observed with polyclonal antibodies coating the wells was surprising. The polyclonal antibodies may capture M-protein but prevent access to the epitope seen by the monoclonal antibodies used as 'sandwich'; lack of access to the epitope could occur if the polyclonal capture antibodies react with more than one site on the same molecule of M-protein.

C. Increase in Level of Triton X-100

Triton X-100 could be increased to levels as high as 1.2% with an enhancement of approximately 40% in absorbance relative to the standard use of 0.09% at the stage of incubation of coated plates with the solution containing virus (see Figure 4). The additional Triton X-100 evidently helps to open up the virus and expose M-protein for capture by the antibody-coated wells of the Microtiter plate. The higher level could be used provided that the additional Triton X-100 above 0.09% was added after the sample was aliquoted into the well of the coated Microtiter plate. Direct addition of the higher level of Triton X-100 to the well before the sample resulted in lower readings perhaps due to solubilization of capture antibody.

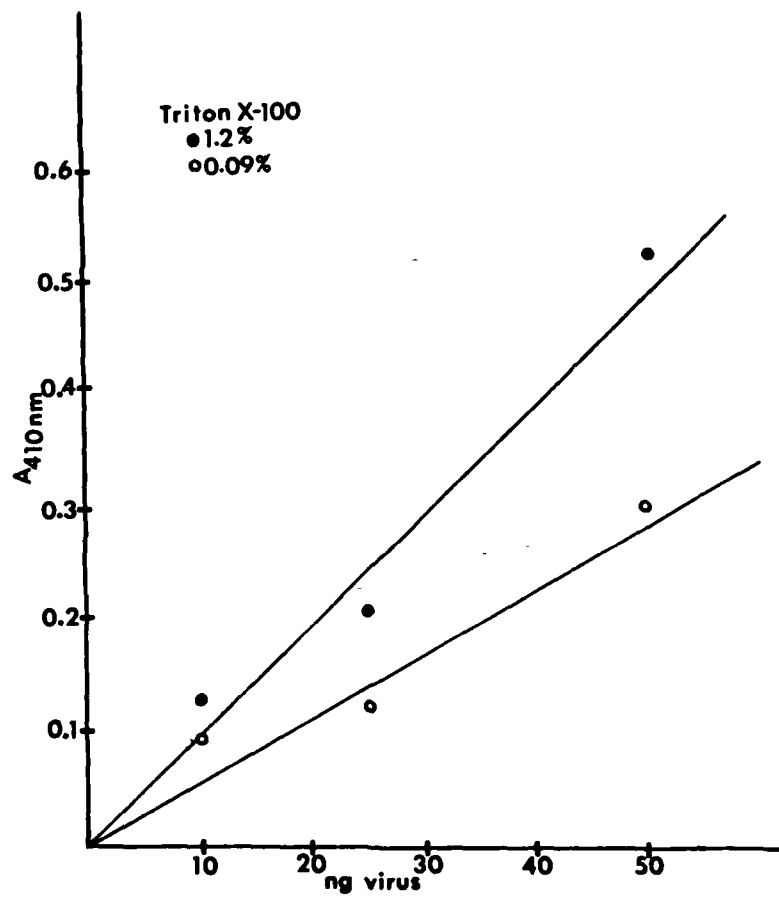


Figure 4. ELISA assay of X-53a virus standard in the presence of 1.2% Triton X-100 (●) or the lower level, 0.09% Triton X-100 (○).

### III. Analysis of Clinical Specimens

Clinical specimens from Mount Sinai, Nassau County Medical Center and from Dr. Meikeljohn's laboratory at the University of Colorado at Denver were analyzed by ELISA assay. These clinical specimens represented three different collection techniques in various media; nasal wash in saline at Mount Sinai, throat and/or nasopharyngeal swab in viral transport media at Nassau County and gargle in veal infusion broth-gelatin at Denver. In all cases type A influenza virus containing specimens had a higher reactivity than those specimens from which no isolate was made. Furthermore, it is highly likely that influenza virus antigen may be present in a number of the negative specimens, even though no isolate could be made; thus, the negative specimens may have a higher mean reactivity than if all specimens were negative for antigen as well as viable virus.

#### A. Mount Sinai Specimens

Clinical specimens were assayed as collected during the influenza season according to the optimized ELISA protocol with plates coated with 2B-B10-C12 and 1G8-A11 (see p.9). Unfortunately, both types A (H3N2) and B influenza viruses circulated with an overall lower rate of isolation than in the previous season.

As a result, only three type A influenza virus isolates were made from the 81 nasal wash specimens collected from children at Mount Sinai.

The relative ELISA values (ratio of absorbance of specimen/absorbance of background) for the three specimens which were positive for type A influenza virus were 1.11, 1.10 and 1.06. These values were slightly above the mean of 1.00 for the 66 isolate-negative specimens assayed directly in ELISA.

#### B. Nassau County Medical Center Specimens

Nassau County Medical Center specimens were assayed according to the optimized protocol before freezing of specimens. The relative absorbance values were determined for all type A Influenza containing specimens, type B specimens and a set of isolate-negative specimens collected on 3/14/86 at the end of the influenza season (see Figure 5). The median was slightly higher for the type A positive specimens than the type B or negative specimens with a median of 1.07 for type A, 1.04 for type B and 1.03 for the negative specimens. The means were 1.08 for type A, 1.03 for type B and 1.06 for negative specimens.

ELISA Analysis of Influenza containing Specimens  
Optimized Protocol (11/85)

1. Coat plates with 200  $\mu$ l carbonate buffer containing purified IgG (2 to 5  $\mu$ g/100  $\mu$ l).
2. Wash 3 times with 200  $\mu$ l PBS-Tween.
3. Postcoat with 200  $\mu$ l of PBS-Tw containing 0.5% BSA. Incubate for one hour at room temperature.
4. Fill row 2 with 100  $\mu$ l of PBS-Tw with additional 0.18% Triton X100. Fill remaining wells with 100  $\mu$ l of PBS-Tw with additional 0.09% Triton X100.
5. Freeze-thaw specimen 3 times and warm in 37<sup>o</sup>C water bath.
6. Add 100  $\mu$ l of specimen to row 2 and dilute in two-fold increments through row 12.
7. Incubate at 37<sup>o</sup>C for 1/2 hour.
8. Wash 3 times with 200  $\mu$ l PBS-Tween.
9. Add 100  $\mu$ l rabbit anti-M IgG at a concentration of 4  $\mu$ g/ml in PBS-Tw and 0.5% BSA to all wells. The IgG was absorbed prior to use (see footnote<sup>a</sup>).
10. Incubate for one hour at room temperature.
11. Wash 3 times with 200  $\mu$ l PBS-Tween.
12. Add 100  $\mu$ l of goat anti-rabbit IgG conjugated with alkaline phosphatase at a dilution of 1:750 in PBS-Tween and 0.5% BSA<sub>b</sub>. The conjugate was adsorbed prior to use (see footnote<sup>b</sup>).
13. Incubate for one hour at room temperature.
14. Wash 3 times with 200  $\mu$ l PBS-Tween.
15. Add 100  $\mu$ l p-nitrophenyl phosphate substrate in diethanolamine buffer.
16. Incubate for one hour at room temperature in dark.
17. Stop reaction with 100  $\mu$ l 1N NaOH and read absorbance with Titertek.

- a. IgG is diluted to one ml with PBS-Tw and 0.5% BSA. One ml 'control' gargle specimen is added and held at 5-10° for 15 min. IgG is then diluted to final concentration of 40 µg/ml; 3% allantoic fluid added, and held an additional 15' at 5-10°. IgG preparation is clarified by centrifugation at 1500 rpm for 30 min.
- b. Conjugate is diluted to 0.5 ml with PBS-Tw and 0.5% BSA and 0.5 ml 'control' gargle added. Conjugate is held at 5-10° for 15 min. and clarified by centrifugation at 1500 rpm for 20 min. The conjugate is then diluted to the final concentration.

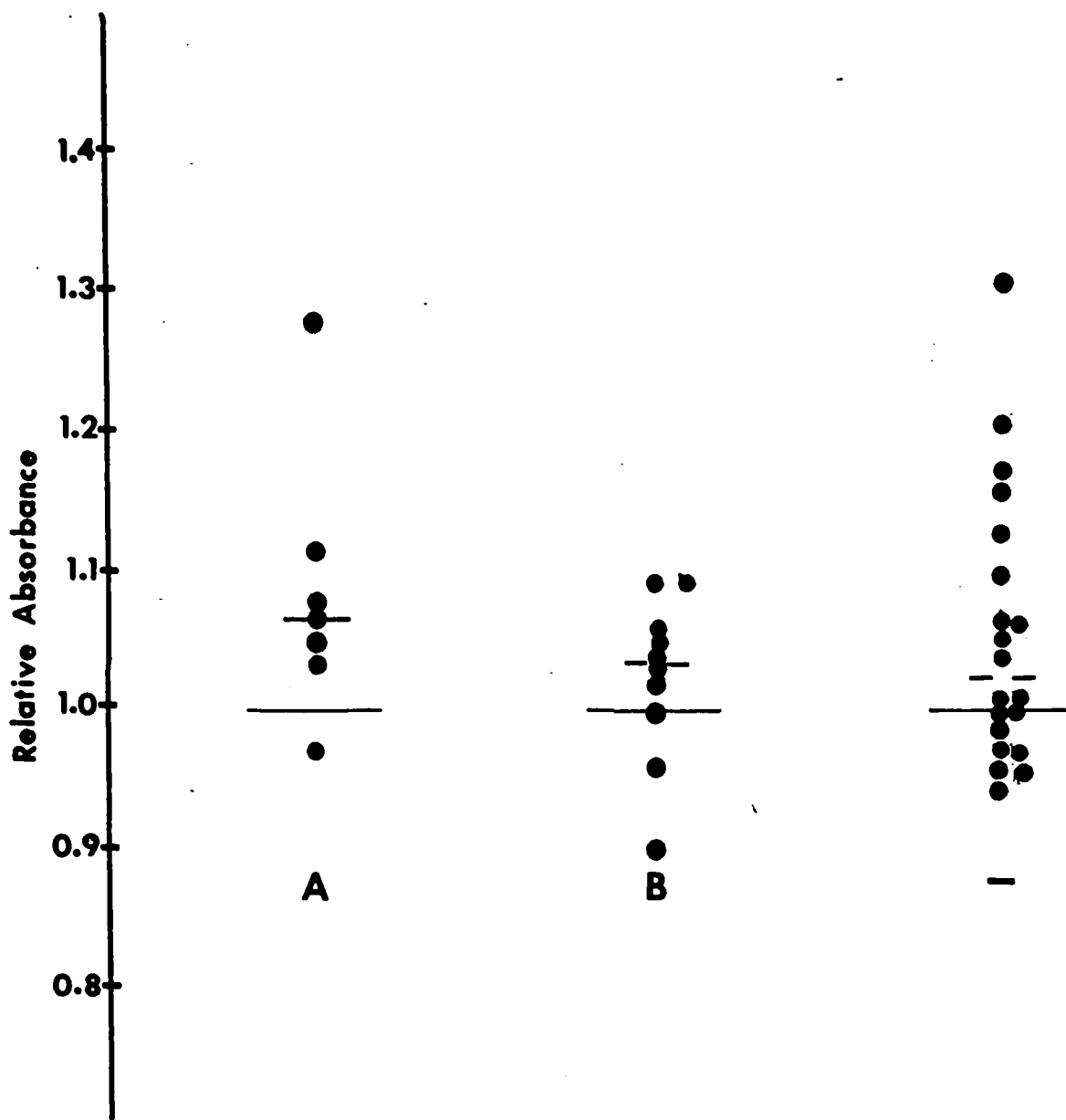


Figure 5. Specimens obtained from Dr. Steve Lipson of Nassau County Medical Center for the 1986 winter influenza season. Specimens were positive for type A, H3N2 (A), type B (B) or no isolate was made (-). Bars denote medians.

C. University of Colorado at Denver (Dr. Meikeljohn)

A set of type A (H1N1) positive specimens and negative specimens (negative by isolation and serology) obtained from Dr. Meikeljohn's laboratory were analyzed by ELISA assay. These specimens were collected during the winter of 1983-1984 through their surveillance of Lowry AFB. The specimens were obtained by having individuals with influenza-like symptoms gargle a veal infusion broth-gelatin mixture and collect the results.

Specimens were assayed according to the optimized protocol. (see Figure 6). The positive specimens had a median of 1.12 and a mean of 1.15; negative specimens had a median of 1.03 and a mean of 1.03.

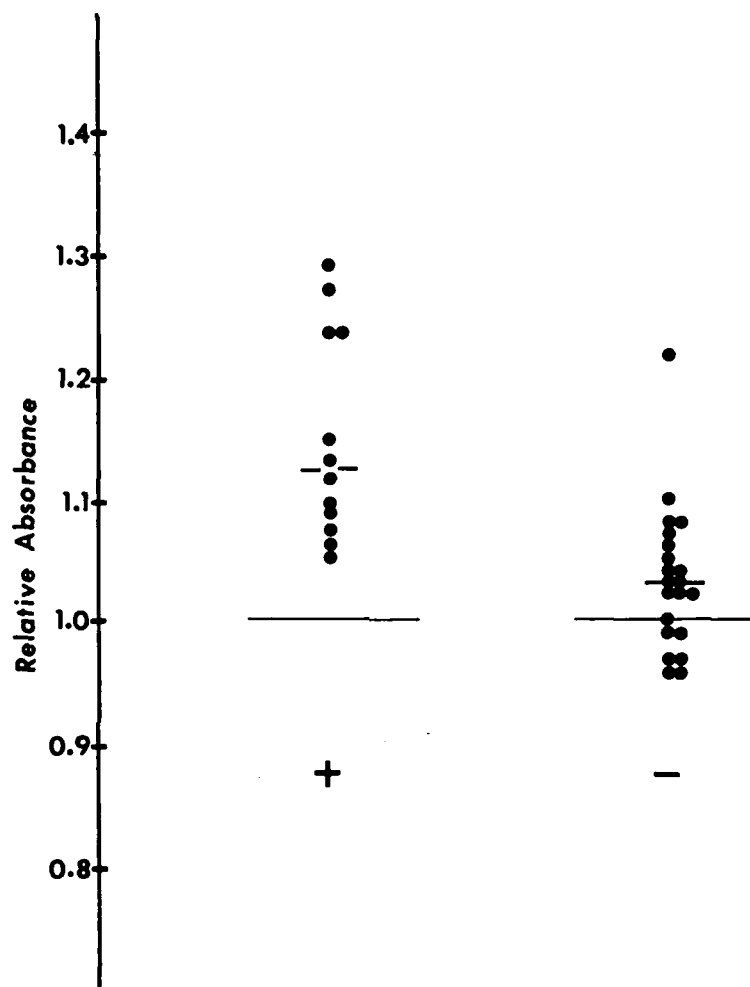


Figure 6. Specimens (H1N1) from Lowry AFB obtained from Dr. Meikeljohn at the University of Colorado at Denver from the 1983-84 winter influenza season. Specimens were analyzed according to the optimized protocol. Specimens were either positive for type A influenza, H1N1 (+) or negative both by isolation and serology (-). Bars denote medians.

D. Typing influenza virus clinical isolates through ELISA assay of M-protein

The ELISA system for detecting influenza viruses through M-protein may have an important application for typing influenza virus isolates from clinical specimens. Eight specimens from which influenza virus isolates were made were amplified in eggs and the allantoic fluids were analyzed by ELISA at four-fold dilution. These specimens were compared with eight specimens which were isolation negative for influenza virus (see Figure 7). All specimens were collected during the 1984-85 influenza season. All positive specimens were reactive in the ELISA assay, negative specimens did not react. No centrifugation or any other pretreatment was used before assay.

Virus isolates amplified in MDCK cells were also analyzed by the ELISA assay. Early isolates from the 1984-85 season showed high reactivity, later isolates from that season and from 1986 had very low reactivity (see Figure 8).

These results may indicate that the epitope on M-protein recognized by our monoclonal antibodies 2B-B10-C12 and 1G8-A11 may have mutated or that the gene for another M-protein may have been introduced into the circulating H3N2. Our standard H3N2 (15-2) isolated from a clinical specimen last year showed high cross-reactivity with M-protein from X-53a. If the epitope associated with the 1986 H3N2 has been altered, this would explain the relatively low degree of positivity seen in our direct assay of clinical specimens.

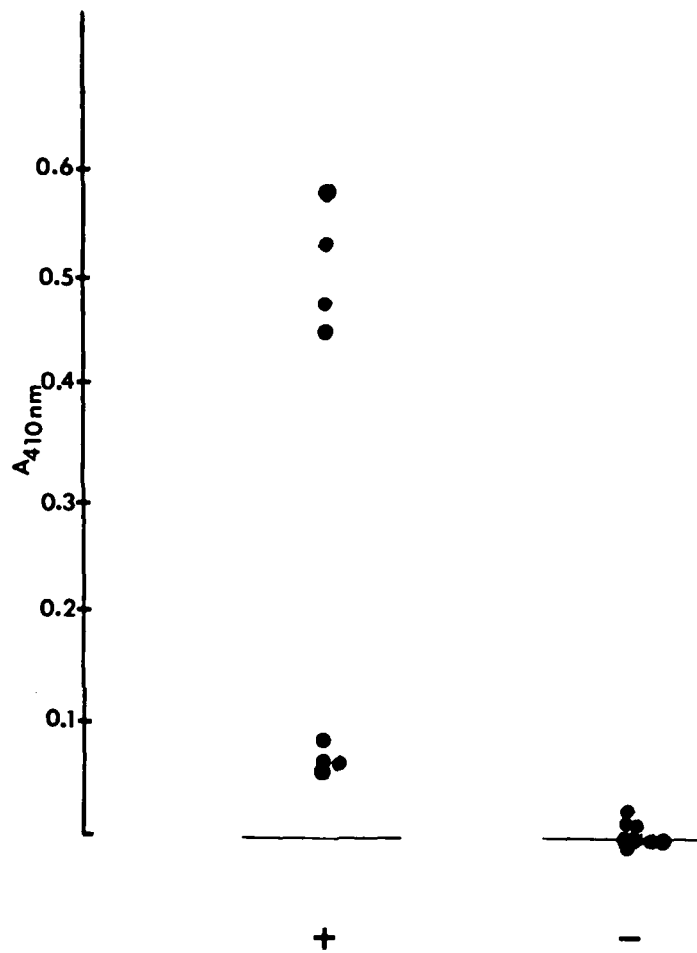


Figure 7. ELISA reactivity of specimens from the 1984-85 winter influenza season amplified in eggs and assayed at a 1:4 dilution. Specimens were either positive for type A influenza virus, H3N2 (+) or no isolate was made (-).

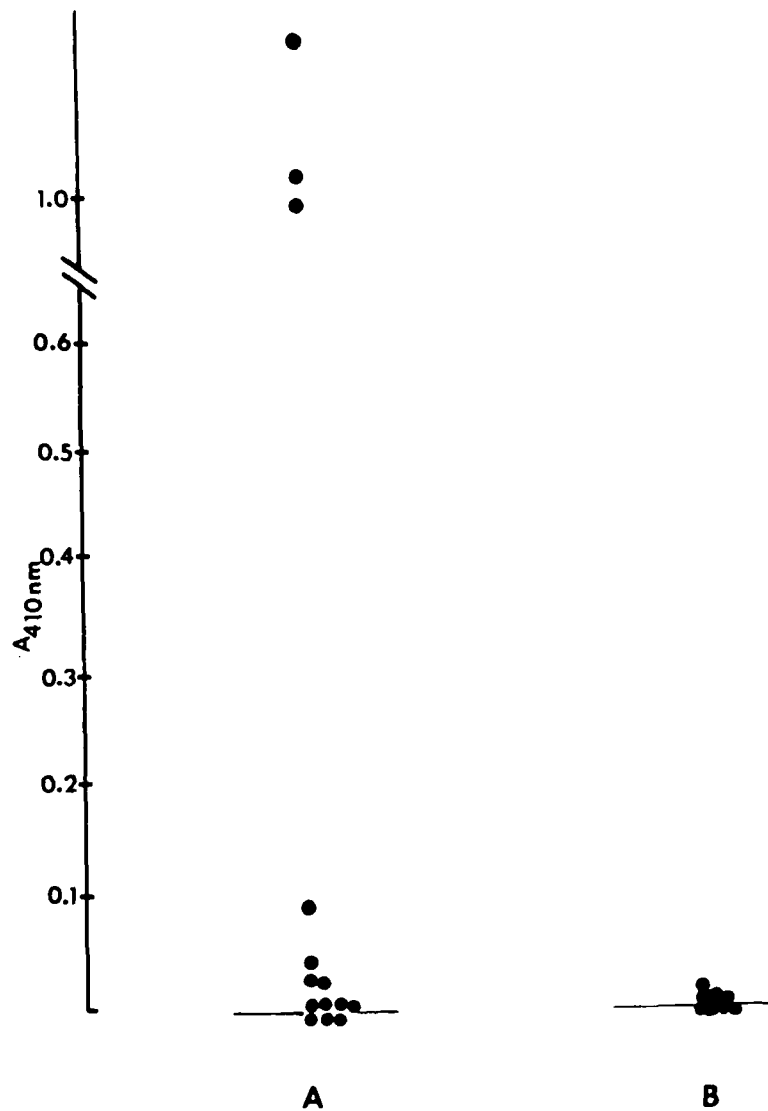


Figure 8. ELISA reactivity of specimens from 1984-85 and 1985-86 winter influenza seasons. Type A (H3N2) (A) and type B (B) influenza virus containing specimens were amplified in MDCK cells and then analyzed. Higher reactivity was seen among the early isolates from the 1984-85 season.

#### IV. New Hybridoma Lines Secreting Antibodies to M-protein

Several new hybridoma lines have been generated which secrete monoclonal antibodies to M-protein. Lines of interest include 611-B12 (IgG<sub>2a</sub>), 611-G10 (IgM), 951-C4 (IgG<sub>1</sub>) 963-D3 (IgG<sub>1</sub>) and 961-G8 (IgG<sub>1</sub>)<sup>2a</sup> all of which have strong reactivity with M and X-53a virus, 951-D10 (IgG<sub>2b</sub>) strong reactivity with M-protein and weak reactivity with X-53a<sup>b</sup> virus; 961/6 (IgG<sub>1</sub>) reactive with M-protein but not with X-53a virus; and 953-G6<sup>1</sup> (isotype not determined) reactive with a synthetic peptide of M-protein sequence (amino acids 83-100).

Ascites fluids have been prepared for each of these lines. Several of these lines react well in immunofluorescence localization of M-protein in infected cells. These lines include; 611-B12, 611-G10, 961-G8-H3, 963-D3-G10 and 951-C4-G2. Monoclonal antibodies are being purified for each line.

Hybridoma lines were prepared by fusion of spleen cells from Balb/c mice immunized with M-protein followed by a boost with the synthetic peptide conjugated to KLH and a second boost with a mixture of M-protein and synthetic peptide (free and conjugated).

A summary of hybridoma cell lines secreting monoclonal antibodies to M-protein which forms our current panel is presented in Table 1.

Table 1

## HYBRIDOMA LINES SECRETING ANTIBODIES TO M-PROTEIN

	<u>ISOTYPE*</u>	<u>X10<sup>-3</sup></u> <u>M-PROTEIN</u>	<u>X10<sup>-3</sup></u> <u>X-53a</u>	<u>M-protein/X-53a</u> <u>RATIO</u>
2B-B10-C12	IgG <sub>1</sub>	5200	4300	1.2
2B-B10-F1	IgG <sub>1</sub>	2200	1500	1.5
2B-B10-A5	IgG <sub>1</sub>	498	186	2.7
2B-B10-G9	IgG <sub>1</sub>	2200	1600	1.4
2E5-C1	IgG <sub>1</sub>	5475	3900	1.4
2E5-D4	IgG <sub>1</sub>	4933	1917	2.6
6B9-B8	IgG <sub>1</sub>	4850	48	101.0
6B9-G3	IgG <sub>1</sub>	5800	11	527.3
3G12-C12	IgG <sub>1</sub>	5800	1600	3.6
9E8-B2	IgG <sub>1</sub>	280	120	2.3
1G11-D11	IgG <sub>1</sub>	3749	729	5.1
1G8-A11	IgG <sub>1</sub>	2187	647	3.4
1G8-H9	IgG <sub>1</sub>	609	336	1.8
611-G10-D3	IgM	243	59	4.1
611-B12-D10	IgG <sub>2a</sub>	207	101	2.0
951-C4-G2	IgG <sub>1</sub>	1150	370	3.1
963-D3-G10	IgG <sub>1</sub>	3700	2600	1.4
961-G8-H3	IgG <sub>1</sub>	1450	1250	1.2
951-D10-B3	IgG <sub>2b</sub>	2150	1000	2.2
961/6-B10	IgG <sub>1</sub>	2200	< 0.3	>7333.3

\* all lines analyzed to date secrete antibodies with kappa light chains.

## V. Combination of Monoclonal Antibodies to Enhance Capture Assay System

A combination of 1G8-A11 and 2B-B10-C12 purified monoclonal antibodies were used as the capture antibodies on polystyrene plates for specimen analysis for the 1985-86 winter influenza season based on earlier results comparing various combinations available at that time. Additional hybridoma lines secreting antibodies to M-protein have been developed in our laboratory and we therefore undertook evaluation of our present collection. Various combinations of purified monoclonal antibodies were adsorbed to polystyrene microtiter plates (Immulon 1) with a total of 4  $\mu$ g monoclonal antibody coating each well in a 200  $\mu$ l volume. Virus (X-53a, H1N1) in quantities of 0, 10, 25 and 50 ng was exposed to the monoclonal antibody coated plates with mild disruption to expose M-protein and the captured M-protein sandwiched with purified rabbit polyclonal antibodies. Values are reported as a ratio relative to background, thus, 2.0 means an absorbance twofold above background, 6.0 an absorbance 6 fold above background, etc.

Results of one set of experiments are shown in figures 9 and 10. Three monoclonal antibodies were coated on polystyrene plates, 611-B12-D10, 611-G10-D3 and 1G11-D11. Nine combinations were used including 2B-B10-C12 and 1G8-A11. The optimal combination was 2B-B10-C12 and 611-G10-D3. 1G11-D11 and 2B-B10-C12 also performed well, however additional experiments with an H3N2 virus showed less sensitivity than the 2B-B10-C12 and 611-G10-D3 combination. Later experiments on epitope analysis supported the validity of both pairs of capture antibodies; 2B-B10-C12, 611-G10-D3 and 1G11-D11 evidently interact with different epitopes on M-protein.

Figure 9 and 10. Relative absorbance using different monoclonal antibodies (or combinations of monoclonal antibodies) as capture antibodies. A total of 4  $\mu$ g monoclonal antibody was used to coat each well of the microtiter plate (Immulon I). The test virus was X-53a (H1N1) analyzed in quantities of 0,10,25 and 50 ng.

Figure "

vs. X-53a (H1N1)

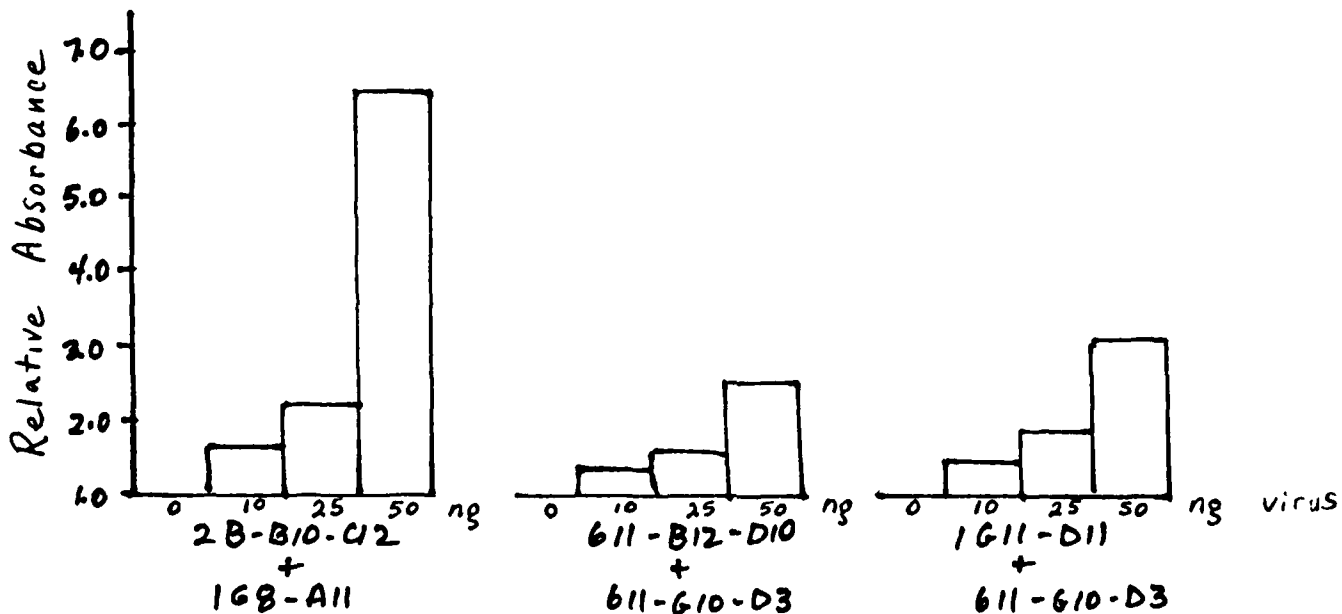
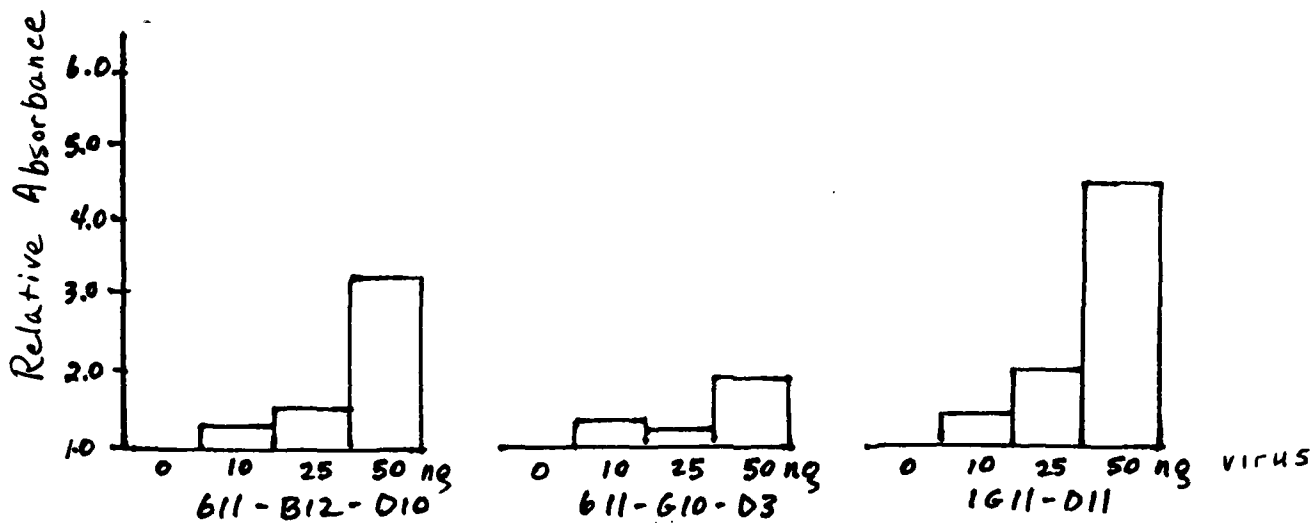
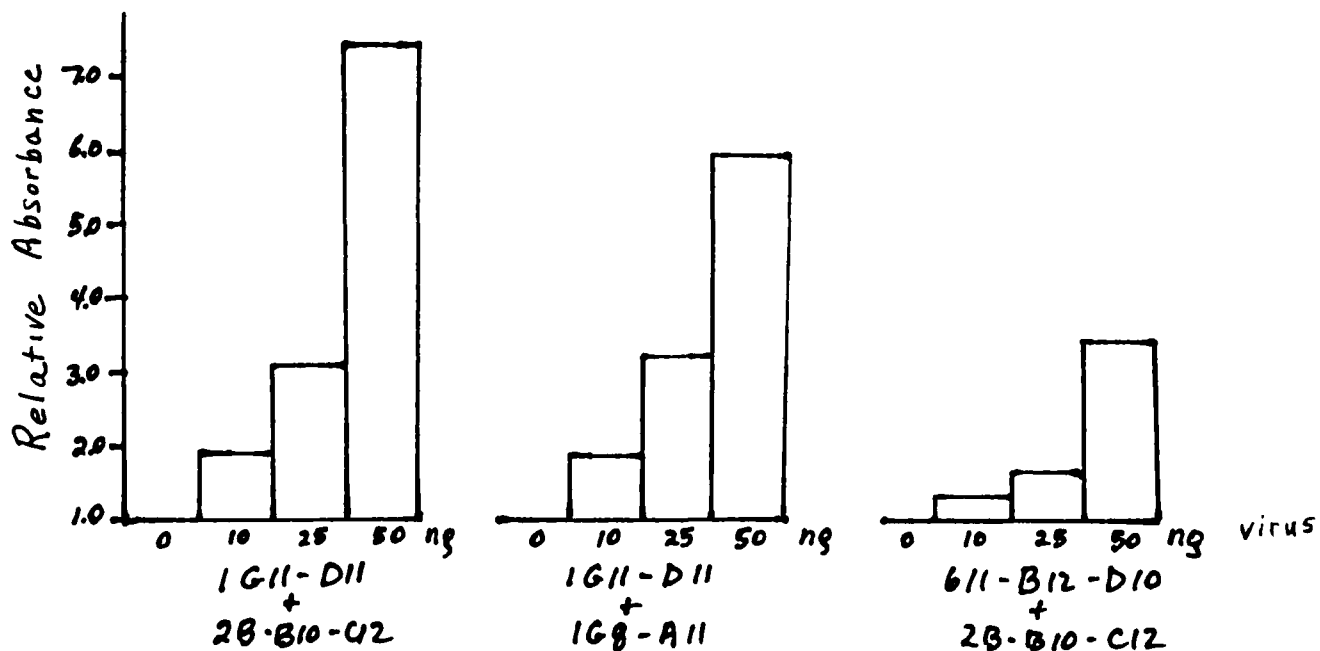
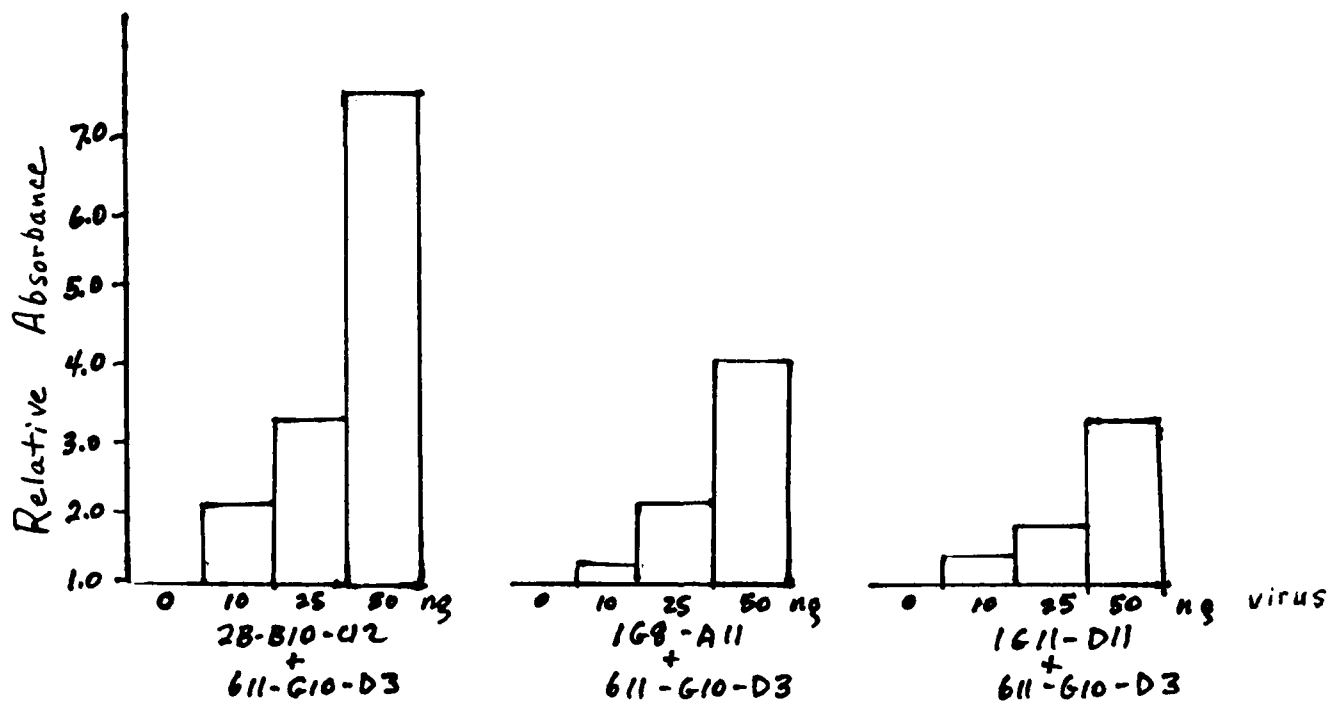


Figure 10

vs. X-53a (H1N1)



## VI. Determination of Epitopes of M-protein

### A. Conjugation of purified monoclonal antibodies with alkaline phosphatase

Monoclonal antibodies were purified by column chromatography and conjugated with alkaline phosphatase according to the protocol of Voller et al (1973) Bull. W.H.O. 53:55 and Kearney et al (1979) J. Immunol. 123:1548. (see protocol in Appendix). Some monoclonal antibodies efficiently conjugated with alkaline phosphatase, others were less effectively conjugated and a third was nearly refractory to conjugation. (see Table 2). The monoclonal antibody which could not be conjugated with alkaline phosphatase, 611-B12-D10, was of the IgG<sub>2a</sub> isotype, the other monoclonal antibodies were IgG<sub>1</sub> or IgM. All had kappa light chains. Conjugation efficiency is dependent on the availability of the appropriate amino acids for linkage with glutaraldehyde. Specific activities obtained are also dependent on the absolute purity of the antibody preparation. Conjugation of polyclonal antibodies resulted in a rather low titer. This probably reflects the fact that only a small proportion of the antibodies even in hyperimmune sera is targeted toward M-protein. Affinity column isolation (with M-protein columns) of antibodies from the polyclonal serum would probably increase specific activity.

Table 2

<u>Conjugated MAb</u>	<u>mg/ml</u>	<u>ELISA ASSAY</u>	
		<u>Titer (3 fold above bkgd)</u>	
		<u>M-protein</u>	<u>whole virus (X-53a)</u>
2B-B10-C12	0.36	15,800	3600
2B-B10-G9	0.50	3,000	770
2B-B10-A5	0.50	5,000	2000
1G8-A11	0.44	20,500	3500
1G11-D11	0.44	10,000	5100
611-G10-D3	0.50	3,000	460
611-B12-D10 (IgG <sub>2a</sub> )	0.40	-	-
M - antisera - rabbit polyclonal	0.71	550	830

## B. Epitope Analysis

Competitive inhibition analysis was performed between alkaline-phosphatase conjugated and non-conjugated monoclonal antibodies by a 'blocking' ELISA assay. The assay was performed by performing a two-fold dilution in duplicate of unconjugated monoclonal antibody (1  $\mu$ g in 200  $\mu$ l PBS-Tween) from row 2 of a 96 well microtiter plate coated with 40 ng/well M-protein. Following incubation and washing steps, monoclonal antibody conjugated with alkaline phosphatase was added at a sufficient concentration to generate 0.4-0.8 absorbance units after one hour incubation. This generally required a dilution of 1:500 to 1:3000 of the conjugated monoclonal antibody. Results of competitive inhibition analysis with 2B-B10-C12 as the conjugated antibody are shown in figures 11 and 12.

Results from competitive inhibition suggest that 1G8-A11 and 2B-B10 (C-12, G9, A5) share the same epitope since 1G8-A11 is capable of nearly complete blocking of 2B-B10 lines and the converse also holds. Monoclonal antibodies which did not block or only partially blocked include 611-G10-D3, 611-B12-D10, and 1G11-D11. Earlier lines including 3G12-C12 and 9E8-G10 totally blocked 2B-B10-C12 and 1G8-A11 and therefore share the same epitope. 2E5-C1 shows partial blockage at a low level and therefore must occupy a site near to that of 2B-B10-C12 but slightly separated.

Based on these results, as demonstrated earlier, 611-G10-D3 is a good choice for use with 2B-B10-C12 (G9 or A5) to maximize coverage of epitopes and produce maximal sensitivity for virus detection. This was also found in practice when combinations were made for virus detection. However, epitope analysis may be difficult to interpret since 611-G10-D3 is an IgM monoclonal antibody. 611-B12-D10 should also be a good choice based on epitope analysis; it shows no blocking activity with 2B-B10-C12 or 1G8-A11. However, in practice, 611-B12-D10 functioned poorly as a partner of either 2B-B10-C12 or 1G8-A11.

1G11-D11 also showed only slight blocking of 2B-B10-C12 or 1G8-A11 suggesting that 1G11-D11 views a different epitope from 2B-B10-C12 or 1G8-A11. Blocking experiments with 1G11-D11 as the conjugated monoclonal antibody further suggest that the epitope seen by 1G11-D11 is different from that of 611-G10-D3 and that this epitope may be a third site. Experiments with combinations of 1G11-D11 and 611-G10-D3 did not show enhanced viral capture activity. However, 1G11-D11 did complement 2B-B10-C12 well in increasing viral capture sensitivity for X-53a (H1N1), but with less success versus 15-2 (H3N2).

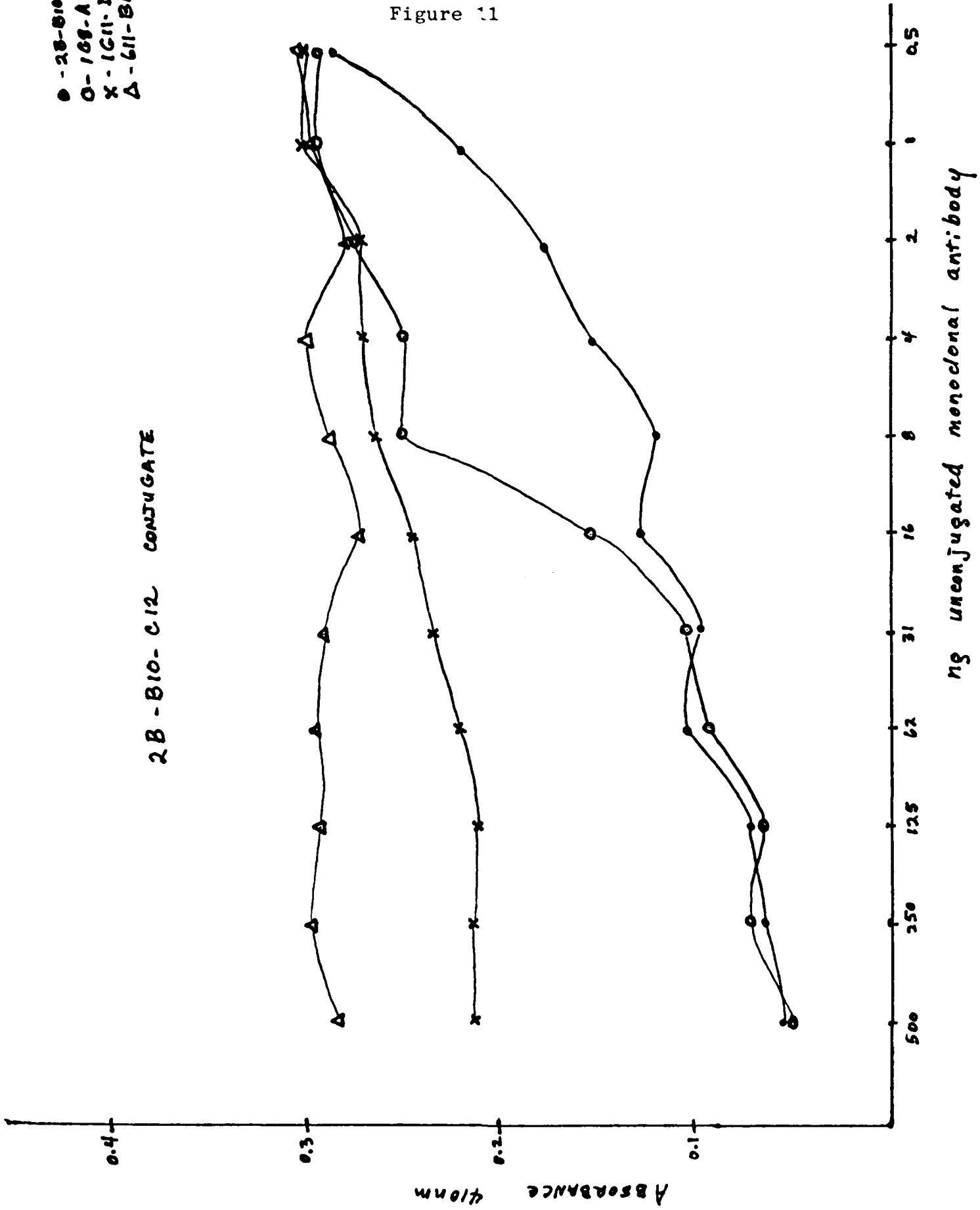
Therefore to date, we have identified 3 epitopes on M-protein which are distinct. Site one includes 2B-B10 (C12, G9, A5), 1G8-A11, 3G12-C12, and 9E8-G10; 2E5-C1 is in the site one region but slightly removed. Site 2 includes 611-G10-D3 and Site 3 includes 1G11-D11. However, 611-G10-D3 is an IgM

Figures 11 and 12. Unconjugated monoclonal antibodies were diluted in a two fold series from row 2 on a microtiter plate starting at a concentration of 500 ng. Alkaline phosphatase conjugated monoclonal antibody, 2B-B10-C12 served as the test antibody at a fixed concentration. Efficient blocking was seen with 2B-B10-C12, 1G8-A11, 3G12-C12 and 9E8-G10. Little or no blocking was seen with 611-B12-D10, 1G11-D11 or 611-G10-D3.

● - 2B-B10-C12  
 ○ - 1G9-A11  
 X - 1G11-D11  
 Δ - 611-B12-D10

Figure 11

2B - B10 - C12 CONJUGATE

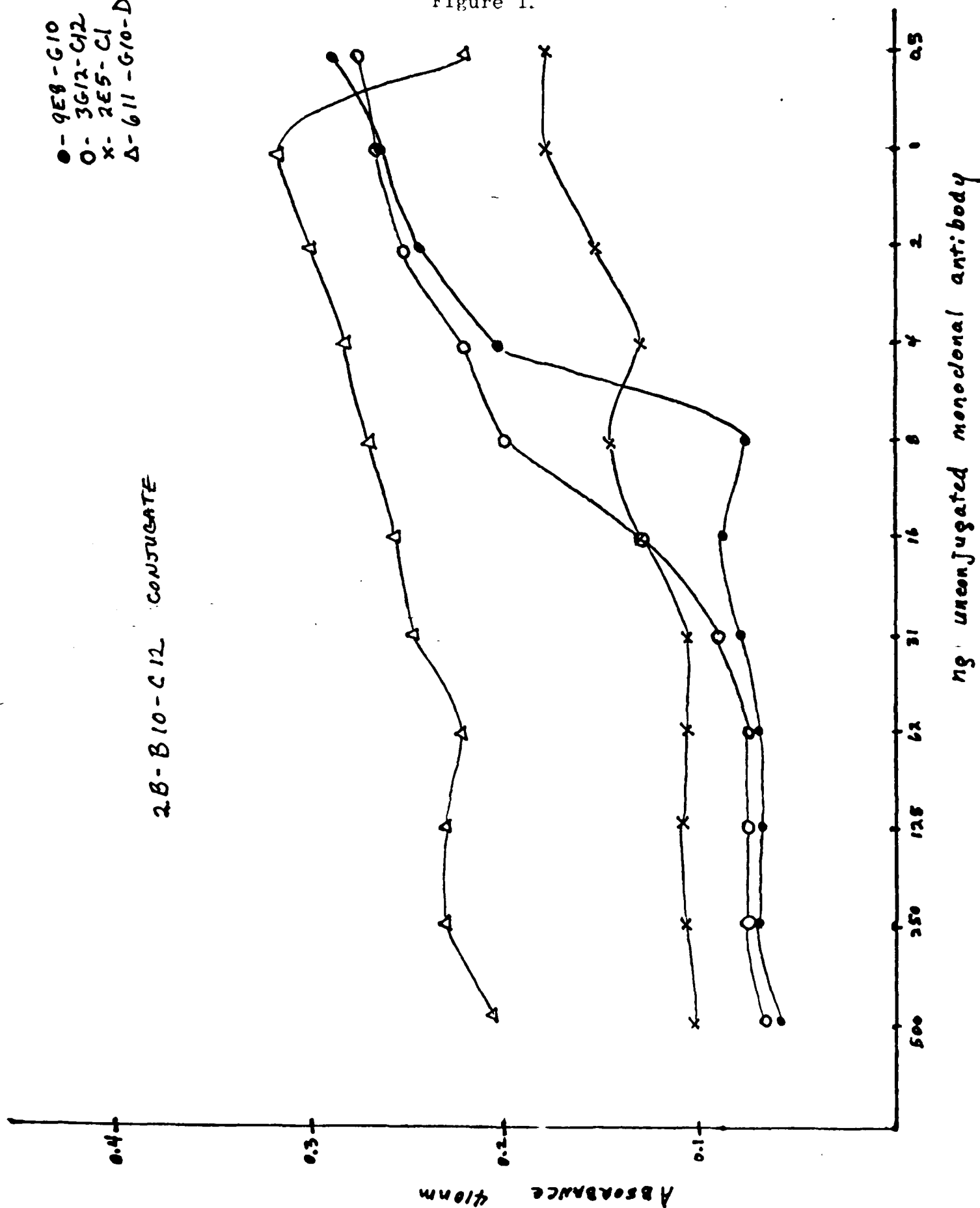


ng unconjugated monoclonal antibody

Figure 13

- - 9E8-G10
- - 3G12-G12
- x - 2E5-C1
- △ - 611-G10-D3

2B-B10-C12 CONJUGATE



ng unconjugated monoclonal antibody

molecule and may cause problems in interpreting competition data.

Analysis of our new hybridoma lines is continuing with purification of antibodies from ascites fluids and conjugation of alkaline phosphatase. Our new panel should provide additional antibodies for mapping the epitopes of M-protein and aid in developing the most sensitive capture antibody system for type A influenza viruses based on M-protein detection.

## VII. Western Blot Analysis

A protocol was developed by Steve Popple for analysis of monoclonal antibodies by the Western blot technique to verify their specificity for M-protein. This technique provides an alternative method in addition to ELISA analysis (following coating of polystyrene plates with M-protein) for detection of monoclonal antibodies reactive with M-protein.

Western blot analysis involves the transfer of proteins to nitrocellulose membranes following their separation by SDS slab gel electrophoresis. Electrophoretic transfer was employed for this procedure. The monoclonal antibodies undergoing analysis are allowed to interact with the 'blotted' membrane following 'blocking' with the appropriate BSA-serum mixture. Binding of monoclonal antibody to specific protein components on the blotted membrane is detected by adding alkaline-phosphatase conjugated sheep (or goat) anti-mouse antibodies and incubating with a substrate (Promega Biotec) which precipitates on cleavage forming an insoluble blue precipitate at the site of enzyme activity and antibody localization. A detailed protocol is provided.

Quantities as low as one microgram total viral protein or 0.5 microgram M-protein were sufficient for electrophoresis, transfer and detection. All monoclonal antibodies which were previously determined to have M-protein specificity by the ELISA assay were confirmed as M-protein specific by the Western blot technique. (See figure 13).

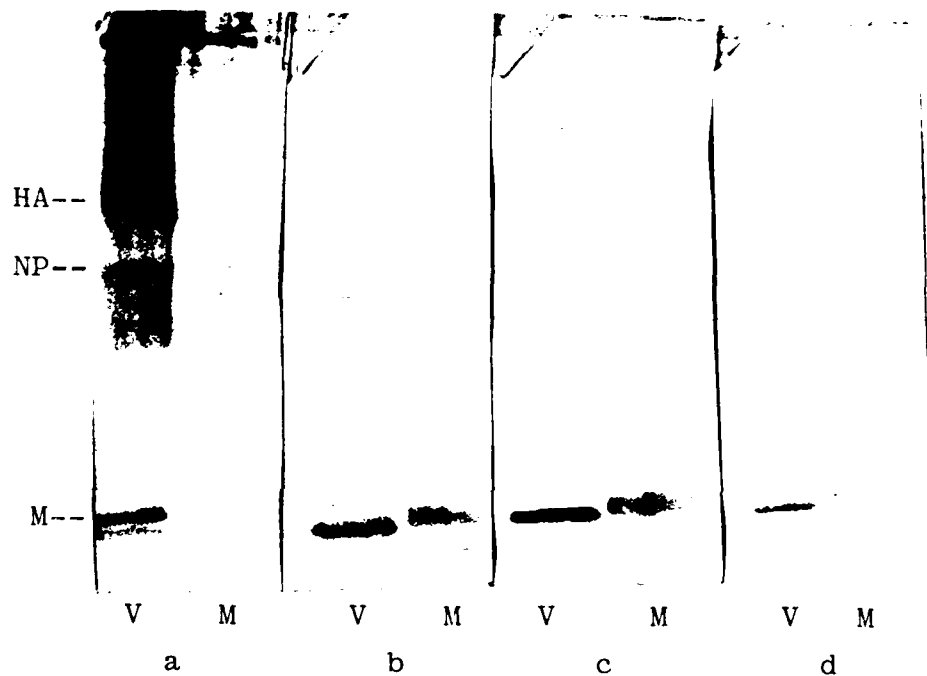


Figure 13. Western blot analysis of total X-53a (H1N1) virus (V) or M-protein (M) (non-reduced) following electrophoresis and electrophoretic transfer. Nitrocellulose strips were incubated with the following antisera or ascites preparations; (a) anti PR/8/34 (H1N1) virus or (b-d) monoclonal antibodies from hybridoma lines directed against M-protein, (b) 2B-B10-G9, (c) 1G11-D11 and (d) 611-G10-D3 developed according to the Western Blot protocol (see appendix).

### VIII. Synthetic Peptides of M-protein

Synthetic peptides can be valuable tools in the development of rapid virus detection systems. They can be used as adsorbents in ELISA detection systems to directly determine the epitope toward which a monoclonal antibody is directed. They can serve in place of viral antigen as adsorbent in virus detection systems based on competitive inhibition. In such a system for viral detection, the peptide would compete with viral antigen in the clinical specimen for the limited amount of antibody added to the specimen. Peptides may also be valuable for use in epidemiologic screening of populations for antibodies to viral components as evidence of recent viral activity.

A peptide was synthesized to a region of M-protein which was determined to have a high probability of being an antigenic site by computer evaluation. Dr. Amrit Judd of S.R.I. International analyzed the M-protein sequence and synthesized the peptide. Mr. Steven Popple, a graduate student in my laboratory, has performed the immunochemical analyses.

83 The peptide represents amino acids 83 through 100  
100  
(A L N G N G D P N N M D K A V K L Y) of the PR8 sequence. Other reported sequences (A/Udorn 72 and A/Bangkok) have an arginine substituted for the lysine at position 95.

The immunoreactivity of the peptide was evaluated by analyzing the ability of preformed antibodies in rabbit hyperimmune sera prepared to whole virus to bind to the peptide in an ELISA system (Khan et al. 1982). Immulon 2 plates (Dynatech) were coated with M-peptide (1  $\mu$ g/well) or M-protein (0.040  $\mu$ g/well) in 0.1 ml carbonate coating buffer for 18 hours. Endpoint determinations were calculated as absorbance values (A410nm) which were three-fold above background absorbance.

Nineteen out of 21 rabbit sera examined show an increase in antibody level to M-peptide #2 following immunization with various type A influenza viruses with a mean-fold increase of 28.1 and a median-fold increase of 6.0 (see Table 3). These results demonstrate that immunization with influenza viruses from a range of subtypes results in production of antibodies which are reactive with synthetic M-peptide #2.

One of the two sera which showed a two-fold decrease in ELISA titer for immune sera to M-peptide (H3N2, A/Texas/1/77 serum) had the lowest rise observed to M-protein, a two-fold increase (see Table 4). At this time, we have not determined the M-protein titer for the second serum (H3N2, X-73).

When the same sera is analyzed for reactivity to M-protein and the peptide, ELISA titers are three to more than thirty times greater than titers for M-peptide (see Table 4). A/PR/8/34 serum which had the highest titer to M-peptide #2, also had the highest titer to M-protein. The preimmune titers were generally higher for M-peptide #2 than for M-protein.

In order to demonstrate that the M-peptide sequence is an epitope for M-protein, absorption studies were carried out with some of the sera. Aliquots of antisera (80  $\mu$ l) were diluted with 220  $\mu$ l PBS-Tween containing 0.5% BSA and 2% allantoic fluid and absorbed with 50  $\mu$ l aliquots of (1) PBS (unabsorbed), (2) virus; 50  $\mu$ g X-53a (3) M-protein; 50  $\mu$ g or (4) M-peptide #2; 40  $\mu$ g M-peptide #2 and 35  $\mu$ g M-peptide #2 conjugated with KLH. Antisera were incubated overnight at 4<sup>o</sup> and vortexed four times at half hour intervals the following morning. The samples were diluted to 500  $\mu$ l for centrifugation in the SW 50.1 rotor (Beckman) at 40,000 rpm for 90 min. The antisera were diluted to 800  $\mu$ l in the PBS-Tween buffer containing 0.5% BSA and 2% allantoic fluid to provide a 10-fold dilution from the original. The ELISA assay was performed as described above.

Following absorption of rabbit sera with virus, M-protein or M-peptide #2, antibody titers to M-peptide #2 decreased (see Table 5). Since M-protein was capable of absorbing antibodies to M-peptide #2, we can conclude that M-peptide #2 contains an epitope which is found in M-protein.

Monoclonal antibodies to M-protein were also tested for their ability to react with M-peptide #2. None of the hybridoma lines tested (representing ten distinct lines) secrete antibodies which are reactive with M-peptide #2.

The use of a synthetic peptide from M-protein, which represents an epitope, may be useful in monitoring antibody response to influenza as described in the paper by Khan and co-workers (1982). Following infection with influenza virus, antibodies to M-protein rise more rapidly than those directed against the hemagglutinin. ELISA assays which use a 'cocktail' of synthetic M-peptides as coating on the solid phase could serve to monitor influenza activity in a community, nursing home or military base.

Synthetic peptides to M-protein may be valuable in the development of rapid virus detection systems for influenza virus based on competition type assays. In this type of assay, a fixed amount of antibody is added to a specimen. Subsequently, the mixture of antibody and specimen is then reacted with a known quantity of antigen on a solid support (i.e. Microtiter plate); the synthetic M-peptide could be used to coat the Microtiter plate as a synthetic M-protein antigen. In the competition type of assay, if virus (containing M-protein) is present in the specimen, the virus reacts with the antibody and decreases the ELISA titer on the Microtiter plate coated with synthetic M-protein antigen.

Detection of viruses by competitive inhibition is limited by the availability of purified viral components. Liozner and co-workers (1983) demonstrated that M-protein could be used for competitive inhibition type detection for influenza virus infection. This peptide could serve as an 'artificial' M-protein for development of diagnostic systems for influenza virus.

Two rabbits have been immunized with the M-peptide linked to KLH for generation of hyperimmune sera. High-titer antisera will permit development of competitive inhibition assays for influenza virus based on the use of this peptide as adsorbent on polystyrene beads or Microtiter plates.

Table 3

Titers of Rabbit Antisera to Matrix Peptide #2 following  
Immunization with Various Type A Influenza Viruses

Subtype <sup>a</sup>	Virus	Serum Titers		Ratio
		Preimmune	Immune	Immune/Preimmune
H1N1	A/NWS/33 <sup>b</sup>	188	6,387	34.0
	A/NWS/33 <sup>c</sup>	110	6,384	58.0
	A/PR8/34	240	18,086	75.4
	A/Swine/Cam/39	657	2,177	3.3
	A/USSR/90/77	2,382	2,855	1.2
	A/Brazil/11/78	1,746	5,223	3.0
	X-53a <sup>d</sup>	2,000	>7,290	>3.6
H1N2	X-7 <sup>e</sup>	671	1,951	2.9
	X-7(F1) <sup>f</sup>	1,084	2,377	2.2
	PR8/HK <sup>g</sup>	237	805	3.4
	PR8/HK <sup>h</sup>	270	3,344	12.4
	PR8/HK <sup>i</sup>	182	7,002	38.5
	PR8/HK <sup>j</sup>	102	7,229	70.9
	PR8/HK <sup>k</sup>	30	6,766	225.5
H2N2	A/RI/57/57	217	774	3.6
H3N2	A/Victoria/3/75	759	6,321	8.3
	A/Texas/1/77	1,298	685	0.5
	X-31 <sup>l</sup>	357	1,707	4.8
	X-73 <sup>m</sup>	4,880	2,116	0.4
	X-79 <sup>n</sup>	894	1,089	1.2
H7N2	X-74 <sup>o</sup>	53	684	12.9
	mean*	818	4,198	28.1
	median*	314	2,616	6.0

\*excluding X-53a titers.

for key to superscripts, see p. 8.

Table 4

Titers of Rabbit Antisera to Matrix Protein following  
Immunization with Various Type A Influenza Viruses

<u>Subtype<sup>a</sup></u>	<u>Virus</u>	<u>Serum Titers</u>		<u>Ratio</u>	<u>Immune</u>
		<u>Preimmune</u>	<u>Immune</u>	<u>Immune/Preimmune</u>	<u>M-pro/M-pep</u>
H1N1	A/NWS/33 <sup>b</sup>	178	>72,900	>409.6	>11.4
	A/NWS/33 <sup>c</sup>	104	>72,900	>701.0	>11.4
	A/PR8/34	810	313,096	386.5	17.3
	A/Swine/Cam/39				
	A/USSR/90/77				
	A/Brazil/11/78				
	X-53 <sup>a</sup> <sup>d</sup>	X300	57,150	>190.5	>7.8
H1N2	X-7 <sup>e</sup>	143	50,957	356.3	26.1
	X-7(F1) <sup>f</sup> -	226	>72,900	>322.6	>30.7
	PR8/HK <sup>g</sup>	188	15,405	81.9	19.1
	PR8/HK <sup>h</sup>	155	24,300	156.8	7.3
	PR8/HK <sup>i</sup>	232	70,956	305.8	10.1
	PR8/HK <sup>j</sup>	153	23,183	151.5	3.2
	PR8/HK <sup>k</sup>	73	35,700	489.0	5.3
H2N2	A/RI/5-57	142	20,889	147.1	27.0
H3N2	A/Victoria/3/75				
	A/Texas/1/77	1010	2,046	2.02	3.0
	X-31 <sup>l</sup>				
	X-73 <sup>m</sup>				
	X-79 <sup>n</sup>				
H7N2	X-74 <sup>o</sup>				

Key to Superscripts (Tables 3 and 4)

- a. subtype with reference to surface antigens, H(HA, hemagglutinin), and N(NA, neuraminidase).
- b. virus passed in monkey kidney cells (serum preparation #2042)
- c. virus passed in monkey kidney cells (serum preparation #2043).
- d. recombinant virus containing A/Swine/NJ/11/76 surface antigens and A/PR8/34 internal antigens (see Ref. 2).
- e. recombinant virus containing HA from A/NWS/33 and NA from A/RI/S<sup>+</sup>/57 (see ref. 3).
- f. recombinant virus containing HA from A/NWS/33 and NA from A/RI/S<sup>+</sup>/57 (see ref. 4).
- g. recombinant virus containing HA from A/PR8/34, Cambridge line, and NA from A/Hong Kong/8/68, X623 II 3a, J.L. Schulman (serum preparation #2071).
- h. see g, serum preparation #2072.
- i. see g, serum preparation #3642.
- j. see g, serum preparation #3643.
- k. see g, serum preparation #3644.
- l. high-yielding recombinant virus strain containing HA and NA from A/Aichi/68 and internal antigens including M-protein from A/PR8/34 (see ref. 2).
- m. high-yielding recombinant virus strain containing HA and NA from A/Bangkok/79.
- n. high-yielding recombinant virus strain containing HA and NA from A/Philippines/82.
- o. recombinant virus containing HA from Heq1/Prague/56 and NA from A/Bangkok/79.

Table 5

Absorption of Immune Rabbit Sera with  
Virus (X-53a), M-protein, and M-peptide #2

<u>Antiserum</u>	<u>Test Antigen</u>	<u>Unabsorbed</u>	<u>Virus</u>	<u>Absorbed</u>	
				<u>M-protein</u>	<u>M-peptide #2</u>
A/PR8/34 (#2048, 3/22/86)	M-protein	>729,000	>729,000	126,209	>729,000
	M-peptide #2	14,728	2,440	1,980	2,552
A/PR8/34 (#2048, 4/11/86)	M-protein	328,764	226,490	236,520	324,910
	M-peptide #2	13,030	11,114	7,609	15,742
PR8/HK (#3642, 4/11/86)	M-protein	233,344	54,000	163,653	207,383
	M-peptide #2	1,827	900	770	1,665
PR8/HK (#3643, 4/11/86)	M-protein	76,729	34,794	28,213	61,650
	M-peptide #2	3,610	858	847	888
PR8/HK (#3644, 4/11/86)	M-protein	69,882	13,615	28,421	47,903
	M-peptide #2	2,275	1,200	875	763

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APPENDIX

### Alkaline Phosphatase Conjugation of Antibodies

Modified from A. Voller, D.E. Bidwell and A. Bartlett (1973) Bull. World Health Organ. 53:55-65 and Kearney et al. (1979). J. Immunol. 123:1548

1. Alkaline phosphatase, Sigma P 5521, 5000 U/5mg protein in crystalline form is centrifuged at 3000 rpm for 10 minutes and the supernatant discarded.
2. One ml purified antibody (2 mg/ml) is added to pelleted alkaline phosphatase.
3. The antibody - alkaline phosphatase mixture is dialyzed overnight at 4°C versus 4 liters PBS, pH 7.4
4. The antibody - alkaline phosphatase mixture is removed from dialysis and 20 µl of 10 glutaraldehyde (EM grade) added. The reaction is allowed to continue for two hours at room temperature with gentle stirring.
5. The preparation is dialyzed versus 4 l. PBS, pH 7.4, overnight at 4°C.
6. The preparation is dialyzed a second time versus 4 l. 0.5 M Tris-HCl, pH 8.0.
7. The sample is brought up to 4.0 ml with 0.5 M Tris-HCl containing 0.001 M Mg, Cl<sub>2</sub>, pH 8.0, bovine serum albumin (RIA grade, Sigma A-7888) added to 1.0%, and sodium azide added to 0.04%.
8. The alkaline phosphatase conjugated antibody preparation is stored at 4°C.

## Western Blot Protocol

As adapted by S. Popple from Promega Biotec System.

### SDS- Polyacrylamide gel electrophoresis -

Polyacrylamide gel electrophoresis of proteins of interest is performed in slab gel electrophoresis system. Protein concentrations applied are in the range of 500 pg to 2 µg.

### Electrophoretic transfer -

Electrophoretic transfer of proteins from gel to blotting membranes is performed in a Biorad electroblotting apparatus at 100 watts/0.36 amps for 4 hrs in Transfer Buffer with cooling coil. Promega Biotec membranes or Schleicher and Shull nitrocellulose (BA83, 0.20 µm pore size) work equally well.

#### Transfer buffer

Trizma (Sigma)	7.5g
Glycine (Sigma)	36.0g
Methanol	500 ml
Distilled water	2000 ml

After electroblotting, nitrocellulose strips can be stored in the Revco in sealed polyethylene bags. Strips must be soaked in TBST buffer (see below) prior to use.

### Western Blot

1. Block strips with 15 ml TBST buffer containing 3% BSA and 2% normal sheep serum for two hours, with gentle shaking. Goat serum can be substituted. Match serum to source of secondary conjugated antibody.
2. Remove blocking solution and add 15 ml test antibody diluted in TBST

containing 1% BSA and 1% normal sheep serum. Incubate for 30 minutes with gentle shaking.

3. Remove test antibody and wash with TBST with vigorous shaking for three sequential washes of five min., 30 min., and 10 min.
4. Add alkaline phosphatase conjugated sheep anti-mouse antibodies diluted in 15 ml TBST containing 1% BSA and 1% normal sheep serum. Incubate for 30 min. with gentle shaking.
5. Remove test antibody and wash with TBST with vigorous shaking for three sequential washes of five min., 30 min., and 10 min.
6. Air dry blotted membrane strips on filter paper.
7. Place strips in 5 ml substrate solution in AP buffer (made according to Promega Biotec specifications). The same substrate preparation can be used for visualization of several strips.
8. Stop reaction by immediately placing in stop solution and place in cold overnight.
9. Air dry filters, mount and save.

Antibody dilutions: monoclonal ascites - 1:5000

polyclonal antisera - 1:2000

alkaline phosphatase conjugate (Sigma) - 1:5000

Solutions

TBST Buffer: 10 mM Tris-HCl, pH8.0  
150 mM NaCl  
0.05% Tween 20

prepare from stock solutions:

10 ml 1 M Tris-HCl, pH8.0  
30 ml 5M NaCl  
0.5 ml Tween 20  
959.5 ml distilled water  
1000.0 ml

AP Buffer: 100 mM Tris-HCl, pH9.5  
100 mM NaCl  
5 mM MgCl<sub>2</sub>

prepare from stock solutions:

100 ml 1 M Tris-HCl, pH9.5  
20 ml 5 M NaCl  
10 ml 0.5 M MgCl<sub>2</sub>  
870 ml distilled water  
1000 ml

for each 3 nitrocellulose strips: 5 ml AP buffer  
33  $\mu$ l NBT  
16.5  $\mu$ l BCIP

Stop Solution: 20 mM Tris-HCl, pH8.0  
5 mM EDTA

Prepare from:

10 ml 1 M Tris-HCl, pH8.0  
25 ml 0.1 M EDTA  
465 ml distilled water  
500 ml

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