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Enhancement of Hemagglutination Inhibition by Complement[1]

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Experiments were conducted to determine the effect of specific antibody and complement on hemagglutination inhibition of influenza virus. It was found that early immunoglobulin M antibody to influenza virus inhibited the hemagglutinating capacity of the virus. When fresh guinea pig serum was added, the inhibiting capacity of the serum was elevated from four- to eightfold. When guinea pig serum was treated with known complement inhibitors, it lost its capacity to enhance hemagglutination. The use of functionally purified complement components indicated that the first, second, and fourth components were necessary and sufficient for enhancement of hemagglutination inhibition by specific antibody to influenza virus.

The role of nonspecific serum factors in the neutralization of viruses has been extensively investigated (2, 3, 9, 22). Few studies, however, concerning the role of so-called nonspecific serum factors in the closely related phenomenon of hemagglutination inhibition (HI) by specific antibody have been conducted. Iwasaki and Ogura (6) working with Japanese encephalitis virus (JEV) indicated that early immunoglobulin M (IgM) antibodies directed against JEV had a higher HI titer in the presence of fresh, normal guinea pig serum than in the absence of added serum. By using influenza virus, mouse antisera against influenza virus, and normal mouse sera, Styk et al. (21) demonstrated the presence of a thermolabile factor in normal mouse serum which was responsible for elevating the HI titer of the immune mouse serum. The investigators claimed that the thermolabile factor present in normal serum was not complement (C), since absorption of mouse serum with zymosan (a cell wall extract of yeast which is known to inactivate hemolytic complement [17] did not significantly alter the ability of the serum to enhance HI. Components of C not affected by zymosan (17), however, might have been responsible for the observed enhancement of HI.

This investigation was undertaken to determine if fresh guinea pig serum would enhance the ability of specific influenza antibody to cause HI, and to define the specific serum factor or factors responsible for the phenomenon. The results indicated that guinea pig C could potentiate HI, and that the first, fourth, and second components of C were responsible for the observed enhancement.

MATERIALS AND METHODS

Virus. Influenza virus strain A_2/Aichi/2/68 was used throughout this study. Virus purified by zonal ultracentrifugation, purchased from Electronucleonics, Inc., Bethesda, Md., was used as the source of immunizing antigen. The preparation contained at least 3×10^{11} particles per ml, 2,500 chicken cell agglutinating units per ml, and 7,000 to 9,000 chicken cell agglutinating units per mg of protein.

Virus used for HI tests was kindly supplied by Robert Waldman, Department of Medicine, University of Florida. The virus was propagated in this laboratory by inoculating the allantoic cavity of 9-day-old chicken embryos with 100 median egg infective doses (EID_{50}) of virus. The allantoic fluid was collected aseptically 48 hr after inoculation, after chilling 18 hr to minimize hemorrhaging, and was stored in glass ampoules at −70 C. Pooled allantoic fluid from infected embryos was found to contain 1×10^8 EID_{50} per ml, when calculated by the Reed-Muench method (18), and 128 hemagglutinating (HA) units per ml. The cryoprecipitation technique described by Reinhard and McGraw (19) was used to partially purify the virus from the allantoic fluid. The cryoprecipitated material was further purified by absorption and elution from chicken erythrocytes (1). The final eluate was one-fourth the original volume, and contained 512 HA units per ml. This eluted

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material was examined under the electron microscope and found to be free from significant amounts of particulate impurities. The virus preparation was then distributed into 1-ml samples and stored at −70 C until further use. No loss of HA titer was noted after storage for 6 months.

Antibody to virus. Purified influenza virus (Electronucleonics, Inc.) inactivated with 0.01% formaldehyde was diluted to a concentration of 10^{10} particles per ml in Hanks balanced salt solution (Difco, Inc., Detroit, Mich.) and mixed with an equal amount of Freund complete adjuvant (Difco). Two milliliters of this preparation was injected subcutaneously into both scapular regions of three 4-kg male albino New Zealand White rabbits. Serial bleedings were taken 3, 5, 7, 10, and 14 days after immunization. The blood was allowed to clot, and the serum was treated with periodate and heated to 56 C for 30 min to remove nonspecific inhibitors of influenza virus (8) and then tested for HI ability.

To partially purify the immunoglobulins, the sera were fractionated by precipitation three times with ammonium sulfate by Kendall's method (7) and resuspended to their original volume in isotonic phosphate-buffered saline, *p*H 7.4. The partially purified immunoglobulin preparations were then applied to a Sephadex G-200 column (Pharmacia Fine Chemicals, Uppsala, Sweden) which had previously been equilibrated with phosphate-buffered saline and were eluted with the same buffer at a flow rate of 10 ml/hr. Five-milliliter fractions were collected, protein was estimated by absorbancy determinations at a wavelength of 280 nm by using a Gilford 2400 Spectrophotometer (Gilford Instruments Inc., Oberlin, Ohio), and the fractions were assayed for HI ability. Fractions containing antibody were pooled and concentrated by dialysis against polyethylene glycol (average molecular weight, 15,000 to 20,000; Matheson, Coleman, and Bell, East Rutherford, N.J.) to the original sample volume. The fractions were subjected to immunoelectrophoresis by using goat anti-whole rabbit serum.

Complement and complement components. Fresh frozen guinea pig serum (Pel-Freeze, Inc., Rogers, Ark.) was used as the source of C. The serum was absorbed by using 3×10^9 particles of influenza virus per ml of serum at 0 C for 30 min. After absorption, the mixtures were centrifuged at $100,000 \times g$ for 60 min to remove any virus-antibody complexes. The absorbed C had a titer of 125 CH_{50} units per ml (11). The absorbed serum was distributed into 1-ml samples and frozen at −70 C until used. The first and the second components of guinea pig complement were prepared in this laboratory by using the methods described by Nelson et al. (15) and Ruddy and Austen (20). All other functionally purified C components were purchased from Cordis Laboratories (Miami, Fla.).

Preparation of reagents functionally deficient in C component activity. To prepare guinea pig serum deficient in certain C components, the following methods were employed. Heating the serum at 56 C for 30 min destroyed C1, C2, and C5 (complement components) activity (10). Treating serum with NH_4OH destroyed C4 (4), and treatment with zymosan (Nutritional Biochemicals, Inc., Cleveland, Ohio) removed primarily C3 (17). The addition of sodium ethylenediaminetetraacetate (EDTA) to serum to a final concentration of 0.04 M prevented binding of C1, C2, and C4 (11).

A reagent deficient in C1 was obtained utilizing the euglobulin-free supernatant fluid resulting from adjustment of the ionic strength of guinea pig serum to 0.04 M at *p*H 7.5 (15).

Hemagglutination inhibition assay. Equal volumes (0.025 ml) of twofold dilutions of early IgM antibody diluted in Veronal buffer (VB) (11), and four HA units of influenza virus in the same buffer were mixed in disposable microtiter plates (Cooke Engineering, Inc., Alexandria, Va.) and incubated at 0 C for 4 hr. One-half volume (0.025 ml) of either guinea pig serum, guinea pig serum functionally deficient in certain C components, or VB was added to the virus-antibody mixture and incubated at 37 C for 60 min. After the incubation period, 0.025 ml of a suspension of fresh, washed guinea pig erythrocytes suspended in VB at a concentration of 2.5×10^7 cells per ml was added. The mixture was shaken, and the cells were allowed to settle at room temperature until HA patterns developed (usually 1 to 1.5 hr). The HI end point was taken as that dilution of antibody which failed to completely inhibit agglutination. Duplicate assays were run, and twofold differences in titer were not considered to be significant.

RESULTS

Antibody purification. The results of gel filtration of ammonium sulfate-precipitated immunoglobulin fractions from five immune rabbit sera are illustrated in Fig. 1. In each case, two distinct protein peaks were found. Antibody activity was confined to the first protein peak in the 3- and 5-day sera, but, as time progressed, antibody activity was found to be associated with both of the protein peaks (days 7, 10, and 14).

The fractions comprising the first peak of antibody from each immunoglobulin preparation were pooled and concentrated as described in Materials and Methods. A single precipitin band which migrated in the IgM region was evident after immunoelectrophoresis of the antibody preparations. Goat anti-rabbit serum was used to develop the immunoelectrophoresis slides.

HA and HI activity of guinea pig serum. Guinea pig serum, before and after absorption with virus, was tested for the presence of inhibitors of myxovirus agglutinins by mixing equal volumes of serial twofold dilutions of serum, 4 HA units of virus, and incubating at 37 C for 1 hr. An equal volume of guinea pig erythrocytes (2.5×10^7 cells per ml) was added, and patterns were allowed to develop for 60 to 90 min.

The HI titer both before and after absorption with virus was 16. The same level of HI was found

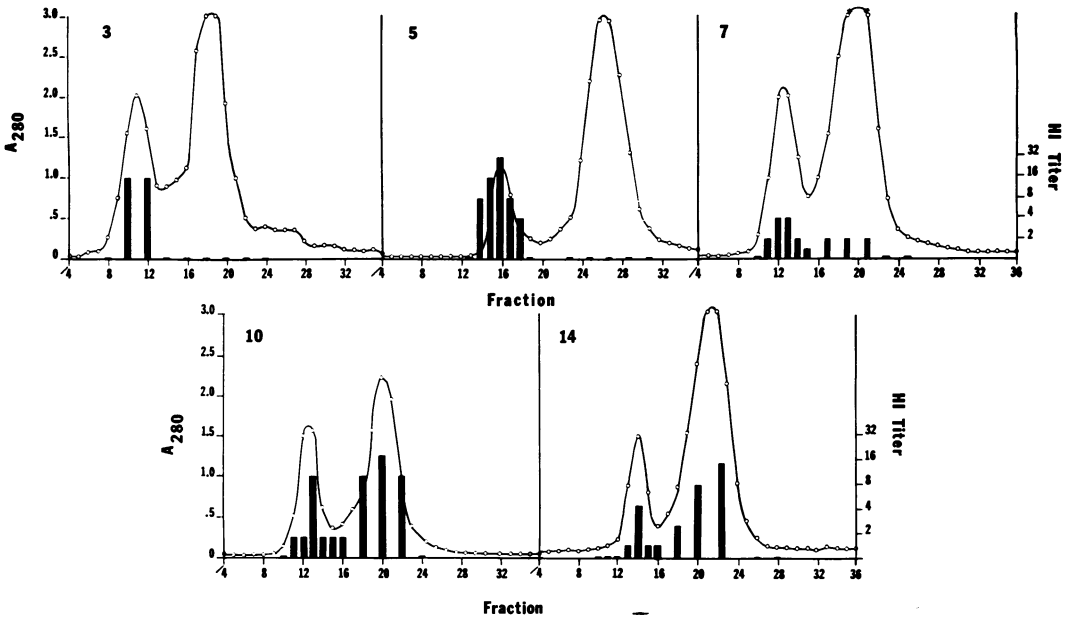

FIG. 1. *Gel filtration of immunoglobulin fractions of rabbit-anti A_2/Aichi/2/68 influenza virus on G-200 Sephadex. The numbers on the upper left-hand part of each graph represent the number of days postimmunization.*

by using guinea pig serum which had been heated at 56 C for 30 min. The absorption would eliminate, to some extent, natural antibodies, and heating would inactivate C, as well as beta inhibitor which is also thermolabile (8). Alpha inhibitor or gamma inhibitor, or both, which are thermostabile and capable of preventing HA of influenza virus strain A_2 (8), may be responsible for the HI ability of normal guinea pig serum. Treatment procedures, however, which inactivate these inhibitors (trypsin, periodate, or neuraminidase) (8) may also inactivate C, and thus these methods could not be used to remove such inhibitors in the guinea pig serum used as a source of C in these studies.

Effect of whole guinea pig serum on HI by early IgM antibodies. To determine the effect of whole guinea pig serum on the ability of early IgM antibodies to inhibit HA of influenza strain A_2/Aichi/2/68, inhibition assays were performed by using dilutions of whole guinea pig serum. The HI titer of anti-influenza antibodies in the absence of added guinea pig serum ranged from 1 to 16 (Table 1). With the addition of various dilutions of guinea pig serum, there was a significant increase in the capacity of the antibodies to inhibit hemagglutination. There was a greater than eightfold increase in HI titer with all preparations of antibody when guinea pig serum at dilutions of 1:10 and 1:20 was present. But, as preliminary results showed, any increase in HI at dilutions of guinea pig serum lower than 1:20 might result from nonspecific inhibitors present in the guinea pig serum. The titer of antibody reacted with 1:40 dilution of guinea pig serum was increased at least eightfold for 3-, 5-, and 10-day sera, and fourfold for days 7 and 14 sera. Similarly, the titers of antibodies reacted with a 1:80 dilution of guinea pig serum increased at least eightfold for day 3 serum, fourfold for day 7, 10, and 14 sera,

TABLE 1. *HI titer[a] of early IgM antibody to influenza A_2/Aichi/2/68 in the presence of guinea pig serum (GPS)*

Dilution of GPS	HI titers on days:				
	3	5	7	10	14
1:10[b]	128	128	128	128	128
1:20[b]	128	128	128	128	128
1:40	128	8	64	128	64
1:80	128	2	64	128	64
1:160	32	1	32	32	8
No GPS	16	1	16	16	4

[a] Reciprocal of highest dilution of antiserum which completely inhibited 4 hemagglutinating units of virus.

[b] The values listed for these dilutions of GPS do not represent true antibody-mediated HI end points, since nonspecific myxovirus inhibitors in the GPS could inhibit HA in all wells.

and insignificantly for day 5 serum. At a 1:160 dilution of guinea pig serum, there was a twofold potentiation of HI with all sera except day 5 serum, which showed no potentiation of HI.

Effect of various complement inactivators on HI potentiation. Guinea pig serum specifically treated (see Materials and Methods) to inactivate certain complement components was tested for the ability to potentiate HI. None of the preparations used were hemolytically active at the test dilution used (1:40), and the results of this experiment are found in Table 2. Zymosan-treated serum caused no significant HI potentiation. Guinea pig serum treated with NH_4OH, with subsequent readjustment of the *p*H to neutrality, caused a twofold increase in HI titer of all sera except day 14. Heat treatment of guinea pig serum at 56 C for 20 min also caused a twofold or less increase in HI titer. EDTA-treated complement was incapable of HI enhancement. Controls showed that the presence of EDTA in the hemagglutination test system had no adverse effect on the viruses' ability to agglutinate the guinea pig erythrocytes.

Serum deficient in C1 was tested for its ability to potentiate the HI ability of the antibodies tested. No significant increase of HI titer was noted upon addition of this treated serum to the virus-antibody mixture. Replenishment of the reagent with $C\bar{1}$ resulted in increased HI titers when mixed with the day 3, 10, and 14 antiserum.

Effect of functionally purified guinea pig complement components on the HI ability of early IgM antibodies. To determine more directly if C was indeed the factor in guinea pig serum which enhanced HI of early IgM antibody, functionally purified guinea pig C components were used. Since $C\bar{1}$ (functionally purified complement component) in the fluid phase can inactivate C4 and C2 prior to their attachment to an immune complex (12, 13), dilutions of $C\bar{1}$ were incubated with the virus-antibody mixture at 0 C for 1 hr prior to addition of the remaining components. Twofold dilutions of $C\bar{1}$ in VB were made in an attempt to find the optimal concentration for HI enhancement and to reduce the amount of activated $C\bar{1}$ in the fluid phase. The addition of a mixture of C4 and C2 to the virus-antibody-$C\bar{1}$ complex (VAbC$\bar{1}$) was followed by incubation at 30 C for 30 min. After the addition of a mixture of C3 through C9, the plates were incubated at 37 C for 60 min to allow the reaction to go to completion. Guinea pig erythrocytes were added (2.5×10^7 cells per ml) and HA patterns were read after 90 min. Table 3 contains the results of experiments carried out on a representative serum (day 10).

The results indicated that all nine components of guinea pig C were not necessary to enhance HI of day 10 serum. The presence of $C\bar{1}$ alone in various dilutions did not enhance the HI titer of the antibody. When C4 and C2 were added in the presence of the highest concentration of $C\bar{1}$, there was a slight suppression of the HI titer. Upon dilution of $C\bar{1}$, the HI titer rose from 8 to 128 when C4 and C2 were added. When all nine components were added, there was a significant increase (over HI levels seen with $C\bar{1}$ alone) in the HI titers of the mixtures containing diluted $C\bar{1}$, but only a twofold increase in the mixture containing undiluted $C\bar{1}$. This implied that there might have been an excess of $C\bar{1}$ in the fluid phase which inactivated some C4 and C2 prior to their binding to the virus-antibody complexes.

TABLE 2. *HI titer[a] of early IgM antibody to influenza virus in the presence of treated guinea pig serum (GPS)*

Treatment of GPS	HI titers on days:				
	3	5	7	10	14
No GPS	16	1	16	16	8
Untreated	128	8	64	128	64
Zymosan	8	1	16	32	8
NH_4OH	32	2	32	32	8
EDTA	16	1	16	16	8
Heat	32	2	16	32	8
C1-deficient serum	16	2	16	32	8
C1-deficient serum + $C\bar{1}$[b]	128	ND[c]	ND	128	64

[a] Reciprocal of highest dilution of antibody which completely inhibited 4 hemagglutinating units of virus.

[b] $C\bar{1}$ euglobulin precipitate obtained by preparing C1-deficient serum.

[c] Not done.

TABLE 3. *Effect of functionally purified guinea pig complement components on HI titer[a] of day 10 IgM antibody to influenza A_2/Aichi/2/68*

Dilutions of complement $C\bar{1}$	HI titers in the presence of complement components:		
	$C\bar{1}$[b]	C1, C4, C2[b]	$C\bar{1}$-C9[b]
Undiluted	16	8	32
1:2	16	128	64
1:4	16	128	128
1:8	16	128	128
1:16	16	128	128
No $C\bar{1}$	16	16	16

[a] Reciprocal of the highest dilution of antibody which completely inhibited 4 hemagglutinating units of virus.

[b] Components.

Day 3 and day 14 sera were also tested in the above manner by using a 1:15 dilution of $C\bar{1}$. Enhancement of HI was found with each of these sera when either $C\bar{1}$, C4, C2, or $C\bar{1}$ through C9 were added (Table 4). In each case, the maximum potentiation of HI by both the mixture of $C\bar{1}$, C4, C2 and all nine individual components was the same as that previously obtained when whole guinea pig serum was used.

An attempt was made to determine if guinea pig C components $C\bar{1}$ and C4 alone could initiate HI enhancement. $C\bar{1}$ at a dilution of 1:15 was added to the virus-antibody mixture as before, various dilutions of C4 or VB were added, and the mixture was incubated for 1 hr at 37 C. Guinea pig erythrocytes were added, and patterns were read after 90 min. The results (Table 5) show that, at the dilution tested, the presence of $C\bar{1}$ plus C4 did not enhance HI. When C2 was added, however, there was an 8- to 16-fold rise in HI titer of all sera tested. The virus-antibody mixture in the presence of C2 alone or C4 alone showed no enhancement of HI. Virus alone with $C\bar{1}$, C4, and C2 was not inhibited from agglutinating the red blood cells; these same components, when mixed individually with the test erythrocytes, did not agglutinate the cells.

It was possible that the $C\bar{1}$, C4, and C2 preparations were contaminated with some later C components (C3 through C9) and that these preparations in combination were not causing HI enhancement on their own merit, but rather were simply contributing additional components to the system. Since C3 must be activated for the other components (C5 through C9) to become activated (5), the $C\bar{1}$, C4, and C2 preparations were tested for the presence of C3 by immune-adherence (16). Dilutions of either C2 or C4 added to sheep $EAC\bar{1}4$ failed to produce immune-adherence when human erythrocytes were added. The addition of both C2 and C3 to $EAC\bar{1}4$ caused immune-adherence to a dilution of 1:128 of C3. $C\bar{1}$ preparations were tested for C3 contamination by using washed $EAC\bar{1}\bar{4}\bar{2}$, and no immune-adherence was found. No detectable amounts of C3 were present in the $C\bar{1}$, C4, or C2 preparations when tested by this sensitive method.

Determination of amount of C2 needed to potentiate HI. Since only undiluted C2 had been used in previous experiments, an attempt was made to establish the minimal dilution of C2 capable of enhancing HI (Table 6).

Undiluted C2 and a 1:4 dilution of C2 in conjunction with $C\bar{1}$ and C4 caused a 32-fold or greater rise in HI titer using the antibody preparations tested. As the C2 preparation was di-

TABLE 4. *Effect of functionally purified guinea pig complement components on HI titer[a] of day 3 and day 14 IgM antibody to influenza A_2/Aichi/2/68*

Components tested[b]	HI titers on days:	
	3	14
$C\bar{1}$	8	4
$C\bar{1}$, C4, C2	128	64
$C\bar{1}$-C9	128	32

[a] Reciprocal of the highest dilution of antibody which completely inhibits 4 hemagglutinating units of virus.

[b] $C\bar{1}$ was diluted 1:15 in all of these experiments.

TABLE 5. *Effect of early reacting guinea pig complement components on the HI titer[a] of early IgM antibodies to influenza virus strain A_2/Aichi/2/68*

Components tested	HI titers on days:			
	3	7	10	14
$C\bar{1}$ (1:15)	8	ND[b]	8	8
$C\bar{1}$ (1:15) + C4[c]	8	8	8	4
$C\bar{1}$ (1:15) + C4 (1:2)	8	8	8	4
$C\bar{1}$ (1:15) + C4 (1:4)	8	8	8	4
$C\bar{1}$ (1:15) + C4 (1:6)	8	ND	8	8
$C\bar{1}$ (1:15) + C4 (1:12)	4	ND	16	4
$C\bar{1}$ (1:15) + C4[c] + C2[c]	128	64	128	64
C2 alone[c]	ND	ND	16	ND
C4 alone[c]	ND	ND	8	ND

[a] Reciprocal of the highest dilution of antibody which completely inhibited 4 hemagglutinating units of virus.

[b] Not done.

[c] Undilute.

TABLE 6. *Effect of dilution of C2 on HI enhancement in the presence of $C\bar{1}$, C4, and C2[a]*

Components tested	HI titers on days	
	3	7
$C\bar{1}$, C4 + GVB[b]	8	<8
$C\bar{1}$, C4 + C2 (undilute)	256	256
$C\bar{1}$, C4 + C2 1:4	256	256
$C\bar{1}$, C4 + C2 1:16	128	16
$C\bar{1}$, C4 + C2 1:64	64	16
$C\bar{1}$, C4 + C2 1:256	16	<8
No C components	8	<8

[a] Reciprocal of the highest dilution of antibody which completely inhibited 8 hemagglutinating units of virus.

[b] $C\bar{1}$ was used at a dilution of 1:15, and C4 was used undiluted in each case.

luted in serial fourfold steps, the HI titer dropped until, at a C2 dilution of 1:256, there was no significant enhancement of HI. The HI-enhancing activity of day 7 serum dropped off more abruptly than that of the day 3 serum.

DISCUSSION

The results obtained in this study with influenza virus corroborate the findings of Iwasaki and Ogura (6), using JEV, that the addition of fresh guinea pig serum to a mixture of virus and antibody caused enhancement of HI by early IgM antibody.

Styk et al. (21) found that, if serum from mice inoculated intranasally or intraperitoneally, or both, with influenza virus was heated at 56 C for 30 min, the HI titer dropped eightfold; if fresh normal mouse serum was added back, the titer returned to the value that it had prior to heating. The authors attributed this effect to the presence of thermolabile antibodies in the mouse serum, but excluded the possibility that complement was the agent responsible for the increased HI titer. Evidence gathered in the present series of experiments, however, has strongly implicated complement or complement components as the factors in guinea pig serum responsible for the enhancement of HI titers of IgM specific for influenza virus.

Treatment of guinea pig serum by several methods which are known to inactivate certain complement components resulted in the elimination of the enhancement of HI produced by the addition of guinea pig serum to virus plus antibody. Thus, the finding that four treatments which are known to inactivate complement components (C1 and C2 with heating; C4 by NH_4OH; C1, C4, and C2 by EDTA; and C3 by zymosan) eliminated enhanced HI strongly supported the concept that complement is indeed the substance in guinea pig serum which enhances HI. In addition, removal of the first component (C1) from guinea pig serum prevented the serum from enhancing HI; subsequent replenishment of this deficient serum with the missing component restored HI enhancement. These findings suggested that all components of hemolytic complement were needed to potentiate HI, and paralleled those findings of Yoshino and Taniguchi (22) with respect to the role of C in the neutralization of herpes simplex virus (HSV). The preparations of various "classical" reagents in both investigations were virtually identical. Although conclusions drawn by extrapolation between different biological phenomena (complement-potentiated virus neutralization and complement-potentiated HI) are, at best, tenuous, the findings of these experiments added to the body of evidence indicating that complement may be responsible for both potentiated neutralization and potentiated HI in many different virus-antibody systems.

With the availability of functionally purified complement components, we felt that it would be possible to elucidate more fully which of the nine complement components were necessary for promotion of enhancement of HI. Findings presented here that $C\bar{1}$, C4, and C2 were necessary and sufficient for potentiation of HI are unique. A role for complement components in virus neutralization has been described by other workers. Linscott and Levinson (9) reported that $C\bar{1}$, C2, C3, and C4 were sufficient for HSV neutralization. Daniels et al. (2) found that $C\bar{1}$ and C4 would neutralize HSV when C4 was present in high concentrations. When C4 concentrations were reduced, potentiation of neutralization required $C\bar{1}$, C2, C3, and C4. The foregoing experiments, however, used a parameter for detecting the effects of C components on a virus-antibody system other than HI.

It was somewhat perplexing to note that treatment of guinea pig serum with zymosan prevented enhancement of HI in this investigation, since this reagent is supposed to adsorb primarily C3 (17). It is known that some quantities of $C\bar{1}$, C4, and C2 are removed by zymosan absorption (14), and it is possible that sufficient quantities of these components were removed to prevent the manifestation of HI enhancement.

Testing of the $C\bar{1}$, C2, and C4 preparations revealed no detectable amount of C3 as measured by the sensitive immune adherence assay (16). If no C3 was present, components which react in sequence after the activation and attachment of C3 would not be activated. It appears, therefore, that the enhancement of HI by early IgM antibody against influenza A_2/Aichi/2/68 may be a biologically active function of the $C\bar{1}$, C4, C2 complex. This has not been reported previously for complement-requiring immune systems. This is not a totally unreasonable finding in view of the many activities in which the components of complement engage. With further investigations of the complement system, it is quite possible that new functions of this system will continue to be encountered.

It must be emphasized, however, that the C component preparations used in these studies had not been purified to molecular homogeneity, so some contaminants in the functionally pure preparations might have been responsible for the enhancement of HI. Whatever the case, the phenomena appeared to be dependent upon the presence of $C\bar{1}$, C4, and C2. Furthermore, C3 was not detectable by immune-adherence in any of

these reagents and if C3 was not present, the remaining components of C (if present in the reaction mixtures) could not become activated. Finally, addition of C3 through C9 to reaction mixtures did not increase the levels of HI beyond those found when just $C\bar{1}$, C4, and C2 were present.

LITERATURE CITED

1. Buckley, P. M., and K. S. Pilcher. 1967. Isolation of ^{14}C-labeled influenza virus from tissue culture, using Ecteola cellulose for final purification. Arch. Gesamte Virusforsch. **21**:454–458.
2. Daniels, C. A., T. Borsos, H. J. Rapp, R. Snyderman, and A. L. Notkins. 1970. Neutralization of sensitized virus by purified components of complement. Proc. Nat. Acad. Sci. U.S.A. **65**:528–535.
3. Daniels, C. A., T. Borsos, H. J. Rapp, R. Snyderman, and A. L. Notkins. 1970. Neutralization of sensitized virus by the fourth component of complement. Science **155**:508–509.
4. Gordon, J., H. R. Whitehead, and A. Wormall. 1926. CXXIX. The action of ammonia on complement. The fourth component. Biochem. J. **20**:1028–1035.
5. Götze, O., and H. J. Müller-Eberhard. 1971. Lysis of erythrocytes by complement in the absence of antibody. J. Exp. Med. **132**:898–914.
6. IWASAKI, T., and R. Ogura. 1968. Studies on complement-potentiated neutralizing antibodies (C'-PNAb) induced in rabbits inoculated with Japanese encephalitis virus. I. The nature of C'-PNAb. Virology **34**:46–59.
7. Kendall, F. E. 1937. Studies on serum protein. I. Identification of a single serum globulin by immunological means. Its distribution in the sera of individuals and of patients with cirrhosis of the liver and with chronic glomerulonephritis. J. Clin. Invest. **16**:921–931.
8. Krizanova, O., and V. Rathova. 1969. Serum inhibitors of myxoviruses. Curr. Top. Microbiol. Immunol. **47**:125–151.
9. Linscott, W. D., and W. L. Levinson. 1969. Complement components required for virus neutralization by early immunoglobulin antibody. Proc. Nat. Acad. Sci. U. S. A. **64**:520–527.
10. Linscott, W. D., and K. Nishioka. 1963. Components of guinea pig complement. II. Separation of serum fractions essential for immune hemolysis. J. Exp. Med. **118**:795–815.
11. Mayer, M. M. 1961. Complement, p. 133. *In* E. J. Kabat and M. M. Mayer (ed.), Experimental immunochemistry, 3rd edition. Charles C Thomas, Springfield.
12. Müller-Eberhard, H. J. 1968. Chemistry and reaction mechanisms of complement. Advan. Immunol. **8**:1–80.
13. Müller-Eberhard, H. J., and I. H. Lepow. 1965. C'1 esterase effect on activity and physiochemical properties of the fourth component of complement. J. Exp. Med. **121**:819.
14. Nelson, R. A. 1958. An alternative mechanism for the properdin system. J. Exp. Med. **108**:515–535.
15. Nelson, R. A., J. Jenson, I. Gigli, and N. Tamura. 1966. Methods for the separation, purification and measurement of nine components of hemolytic complement in guinea pig serum. Immunochemistry **3**:111–135.
16. Nishioka, K. 1963. Measurement of E by agglutination of human erythrocytes reacting in immune-adherence. J. Immunol. **90**:86–97.
17. Pillemer, L., and E. E. Ecker. 1941. Anticomplementary factor in fresh yeast. J. Biol. Chem. **137**:139–142.
18. Redd, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. Amer. J. Hyg. **27**:493–497.
19. Reinhard, K. R., and J. C. McGraw. 1959. Simple means of obtaining concentration of influenza hemagglutinating antigen. J. Bacteriol. **77**:557–558.
20. Ruddy, S., and K. F. Austen. 1969. C3 inactivator of man. I. Hemolytic measurement by inactivation of cell-bound C3. J. Immunol. **102**:533–543.
21. Styk., B., V. Rathova, and D. Blaskovic. 1958. Thermolability of specific haemagglutination inhibiting antibodies against the FE influenza viruses and their reactivation by the addition of fresh serum. Acta Virol. **2**:179–187.
22. Yoshino, S., and K. Taniguchi. 1965. Studies on the neutralization of herpes simplex virus. I. Appearance of neutralizing antibodies having different grades of complement requirement. Virology **26**:44–53.