INACTIVATION OF HUMAN ERYTHROCYTE AGGLUTINOGENS M AND N BY INFLUENZA VIRUSES AND RECEPTOR-DESTROYING ENZYME

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The ability of influenza viruses and receptor-destroying enzyme from *Vibrio cholerae* (R.D.E.) to remove the serologically specific heterogenetic mononucleosis receptor from sheep erythrocytes has recently been reported.[1] It was of interest, therefore, to investigate the effect of influenza viruses and R.D.E. upon serologically recognized receptors of human erythrocytes.

The present paper reports on the selective inactivation of blood group agglutinogens M and N from human erythrocytes by two strains of influenza virus type A —swine influenza virus strain S_{15} and the Melbourne strain—by the Lee strain of influenza virus type B, and also by R.D.E.

MATERIALS AND METHODS

Enzymatically Active Agents.—Influenza viruses were propagated by inoculation into the allantoic cavities of 10-day-old embryonated hen eggs and incubation for 48 hours at 37° C. The infected allantoic fluid was harvested after chilling the eggs. The Melbourne strain of type A, the S_{15} strain of swine influenza virus, and the Lee strain of type B virus, respectively (1×10^8 to 1×10^9 $I.D._{50}$ of virus per ml. of allantoic fluid) were used after purification and four- to eightfold concentration by absorption on and elution from chick erythrocytes. To 50–100 ml. of freshly harvested infected allantoic fluid chilled to 4° C. was added one-tenth volume of washed and packed precooled chick erythrocytes. The mixture was kept 1 hour at 4° C. and repeatedly shaken. The suspension was then centrifuged in the cold, the supernatant fluid discarded, and the cells washed once with cold saline. The packed erythrocytes were resuspended in 15–25 ml. saline, and the virus was eluted at 37° C. during a 10–12-hour period. Subsequently, the chicken erythrocytes were removed by centrifugation, and the virus suspension was used for the enzyme experiments. Occasionally, the virus suspension was reused following additional absorption on and elution from chick erythrocytes after it had acted upon human erythrocytes.

R.D.E. from *Vibrio cholerae* (lot No. 18 Behringwerke, Germany) was employed in concentration of about 200 units/ml. A trace of $CaCl_2$ was added.

Erythrocytes.—Erythrocytes from thirteen different donors possessing the following antigens on their surface were used: (1) O, CDE/e, NN, P; (2) O,cDE/ce, MM, P; (3) A_1, CDe/e, NN, P; (4) B,CDE/Ce, MM, S; (5) O,CDe/e, MN; (6) O,CDE/ce, MM, P, S; (7) O,CDe/ce, NN, P; (8) A_1cde/cde, MM, P, S; (9) A_1B,CDe/e, MM; (10) O,CDe/Ce, MN; (11) A_1B,cDE/c, NN; (12) O,cDE/cE and (13) O,cde/cde, NN, Kk. Wherever possible, erythrocytes homozygous for

the antigen under study were employed. Erythrocytes from citrated human blood of the desired group, stored for less than 1 week, were washed three times with saline and were used as a 2 per cent suspension in all serological experiments. Heparinized chicken erythrocytes, 20–24 hours old, were employed after two washings with saline for the concentration and purification of influenza viruses.

Antisera.—The following sera were procured in this laboratory: human "natural" anti-A and B from blood group O person; human "immune" anti-A from B person; human "immune" anti-B from A person; eel anti-H(O) absorbed prior to use with A_1B cells; human "natural" anti-M; human immune anti-P; and human immune anti-K. The following sera were obtained from Ortho Research Foundation, Raritan, N.J.: one human "immune" anti-B; three human anti-M; one human anti-P; one human anti-S; three rabbit anti-M; three rabbit anti-N; two human anti-D; two human anti-C; two human anti-E; two human anti-c; and one human anti-e. A saline agglutinating anti-S serum was generously donated by Dr. R. E. Rosenfield, Department of Health, New York City.

All agglutinins except anti-c, anti-K, and one anti-S were fully active when tested on red cells in saline suspension. The action of these antibodies was investigated by the indirect Coombs test. The Coombs serum was an antihuman globulin serum (Ortho Lot R7093-L). This diluted serum did not contain demonstrable "T" agglutinins and was used unabsorbed.

All sera containing anti-A or anti-B agglutinins or both were tested against O cells only, unless agglutinogen A or B was being investigated. Except for sera containing anti-A and anti-B agglutinins, no serum contained demonstrable agglutinins against more than one agglutinogen of the intact erythrocyte.

Solutions.—Buffered saline solution (0.85 per cent NaCl, 0.025 molar phosphate pH 7.2) was used throughout. In all enzymatic experiments, both penicillin and dihydrostreptomycin were added in final concentration of 200 units/ml.

Treatment of Human Erythrocytes with Influenza Viruses and R.D.E.—Washed and packed human erythrocytes were added to a fivefold volume of virus suspension and incubated with repeated shaking for 8–20 hours at 37° C. Subsequently, the red cells were separated and washed two to three times with saline at room temperature (22°–25° C.). When R.D.E. was used instead of influenza viruses, similar conditions were maintained, except that the incubation period varied from 2 to 7 hours. In order to observe the influence of prolonged incubation on erythrocyte receptors, tubes containing uninoculated allantoic fluid and erythrocytes to which only buffered saline with antibiotics had been added were included in the studies. Influenza virus preparations and R.D.E. which had been boiled for 30 minutes to inactivate them were also tested.

Absorption of Serum.—An equal amount of serum and packed human erythrocytes treated with virus or R.D.E. as described above was mixed well and kept at room temperature (22°–25° C.) for 2 hours, with frequent shaking during the first 90 minutes. After centrifugation the erythrocytes were discarded, and the serum was used for titration. Eel anti-H(O) serum was absorbed twice. Within each experimental series aliquots of erythrocytes from the same donor treated with the same enzymatically active agent were used for both absorption of sera and titration of agglutinins. Absorption of anti-M and anti-N sera with native MM and NN

erythrocytes was also carried out in order to assess the degree of removal of the corresponding antibodies.

Indirect Coombs Test.—This test was performed according to the procedure given by the Ortho Research Foundation. After incubation of a 2 per cent erythrocyte suspension with incomplete antibody for 1 hour, the red cells were washed three times. The supernatant fluid was removed, 0.1 ml. antihuman globulin serum was added, and the mixture was centrifuged at 1,000 r.p.m. for 1 minute. Readings of agglutination were made with the naked eye. In each Coombs test a positive and a negative control were included.

Titration of Agglutinins.—The method of titration and interpretation of agglutination reactions have been described previously.[1, 2] In the present experiments the volume of each reagent in the test was 0.1 ml. A different pipette was used for each tube in a titration series. Incubation was at about 22° C. except for tests with anti-P, which were incubated at 4° C., and tests with anti-S, which were incubated at 37° C. Agglutination reactions were read with the naked eye at 2 hours and again at 10 hours of incubation after the addition of erythrocytes, except for tests with animal anti-M and anti-N sera, which were read after 30–40 minutes and again after 2 hours of incubation. Each titration included a control of a suspension of intact erythrocytes and also a saline suspension of red cells treated with the enzymatically active agent.

Hemagglutination Inhibition.—The method for studying inhibition of hemagglutination has been described previously.[1, 2] Twofold geometrical dilutions of inhibitor were incubated with four minimum hemagglutinating doses of serum for about 10 hours at 4° C. The appropriate erythrocytes were then added. The results were read after an additional incubation of 2 hours at room temperature (22°–25° C.), except in those instances where animal anti-M and anti-N sera were employed, when the test was read 30–40 minutes after the addition of erythrocytes.

Preparation of Enzymatic Digests from Erythrocytes.—The supernate of enzyme-treated erythrocytes was used either unboiled or after exposure in a boiling water bath for 20 minutes, because M and N agglutinogens are considered to be heat-stable.[3] Insoluble material was separated by centrifugation and tested as a suspension. The suitably concentrated water-soluble material was tested as such and also after dialysis in cellophane casing (Visking, average pore size 48 Å) for 72 hours at 4° C. against 60 volumes of distilled water, which was changed four times.

The color reactions employed in this investigation were the same as those employed previously[1] for the demonstration of the presence of carbohydrate (Molisch and Anthrone reactions) and for neuraminic acid and its derivatives (Morgan-Elson reaction without alkali, and Bial's test, neuraminic acid modification).

RESULTS

Tables 1 and 2 summarize the serological observations. Table 1 shows that treatment of human erythrocytes with swine influenza virus inactivates the antigens M and N, while none of the other blood group agglutinogens tested was demonstrably affected. The Melbourne strain of type A influenza virus and the Lee strain of type B influenza virus affected the erythrocytes in the same way. The preparations of the Lee strain appeared to be weaker in activity especially against N than the strains of type A virus.

TABLE 1

EFFECT OF S_{15} STRAIN OF SWINE INFLUENZA VIRUS UPON BLOOD GROUP AGGLUTINOGENS OF HUMAN ERYTHROCYTES

Serum	Agglutinogen Tested for	Erythrocyte Lot No.	Serum Unabsorbed: Agglutination of Human Erythrocytes* Native	Serum Unabsorbed: Agglutination of Human Erythrocytes* Virus-treated	Serum Absorbed with Virus-treated Erythrocytes: Agglutination of Human Erythrocytes* Native	Serum Absorbed with Virus-treated Erythrocytes: Agglutination of Human Erythrocytes* Virus-treated
Anti-A: Human "immune" (5/57)	A_1	III	256	256	2	4
Anti-A: Human "natural" O person (6780/56)	A_1	III	8	8	<1	<1
Anti-A: Human "natural" O person Spr.	A_1	III	32	32	<1	<1
Anti-B: Human "immune" (12/55)	B	IV	64	64	<1	<1
Anti-B: Human "immune" Ortho 9059-2	B	IV	128	128	<1	<1
Anti-H(O) Eel (1956)	O(H)	V	64	256	2	2
Anti-A & B Human V "natural" O person (6780/56)	A_1B	XI	8	8	<1	<1
Anti-M: Human Ortho R8064	M	VI	512	8	64	<1
Anti-M: Human Ortho T2015	M	VI	32	4	32	<1
Anti-M: Human Cald.	M	IV	32	8	32–64	<1
Anti-M: Animal Ortho 16	M	II	16	4–8	16	<1
Anti-M: Animal Ortho 11, 13	M	IV	8	4	4	<1
Anti-N**: Animal Ortho 6, 9	N	III	16	8	8	<1
Anti-N**: Animal Ortho 12	N	III	16	4	8	1
Anti-M, Anti-N: Human Cald.	MN	V	16	16	16	<1
Anti-M, Anti-N: Animal Ortho 12	MN	V	8	2	8	<1
Anti-S: Human saline Fa 2-214	S	VI	4	4	<1	<1
Anti-S: Human incomplete Ortho	S	IV	8	16	<1	<1
Anti-P: Human Glai.	P	I	4	8	<1	<1
Anti-P: Human Ortho	P	VI	16	16–32	<1	<1
Anti-K Human incomplete Mor.	K	XIII	8	8–16	<1	1
Anti-C: Human Ortho 3080	C	III	8	32–64	<1	1–2
Anti-C: Human Ortho 3080	C	IV	8	16	<1	1
Anti-D: Human Ortho R1208	D	III	4	8	<1	<1
Anti-D: Human Ortho R1207	D	I	8	16	<1	2
Anti-E: Human Ortho R4056	E	IV	8	8	<1	1
Anti-E: Human Ortho R4055	E	II	8	16	<1	2
Anti-c Human incomplete Ortho R 0024, 0027	c	VIII	16	32	<1	<1
Anti-e Human Ortho	e	VIII	16	16	<1	<1

* Expressed as reciprocal of serum dilution.

** "Essentially the same results were obtained with plant anti-N (titer 1:32 at 37° C.) from *Vicia graminea* seeds."

A comparison of the data in the fourth and sixth columns of Table 1 shows that absorption of human sera with virus-treated red cells removes all agglutinins against normal erythrocytes, except those against the antigens M and N, regardless of whether the erythrocytes are homozygous or heterozygous with respect to blood group antigens M and N. The anti-M and anti-N titers against intact erythrocytes are unchanged by absorption with virus-treated red cells (fourth and sixth columns, Table 1). This indicates inactivation of M and N antigens of the virus-treated red cells used for absorption. Untreated MM and NN erythrocytes, on the other hand, removed the homologous antibodies in one absorption. Inactivation of M and N agglutinogens by influenza virus is also demonstrated in the last column of

TABLE 2

EFFECT OF "RECEPTOR-DESTROYING ENZYME" UPON BLOOD GROUP AGGLUTINOGENS OF HUMAN ERYTHROCYTES

SERUM	AGGLUTINOGEN TESTED FOR	ERYTHROCYTE LOT NO.	SERUM UNABSORBED		SERUM ABSORBED WITH R.D.E.-TREATED ERYTHROCYTES	
			Agglutination of Human Erythrocytes*			
			Native	R.D.E.-treated	Native	R.D.E.-treated
Anti-A Human "immune" (5/57)	A_1	III	512	1,024	4	4–8
Anti-A Human "natural" (6780/56)	A_1	III	8–16	16	<1	<1
Anti-B Human "immune" (12/55)	B	IV	128	256	<1	<1
Anti-B Human "immune" Ortho 9059-2	B	IV	128	256	<1	1
Anti-H(O) Eel (1956)	O(H)	VI	64	512	<1	2
Anti-A Human "immune" (5/57)	A_1B	IX	256	256	1	4
Anti-B Human "immune" (12/55)	A_1B	IX	64	64	<1	<1
Anti-M Human Ortho R8064	M	VI	512	8	512	<2
Anti-M Human Ortho T2015	M	VI	64	8	64	<1
Anti-M Human Ortho 16	M	IV	32	16	32	<1
Anti-M Animal Ortho 11	M	IV	32	8	16	<1
Anti-M Human Cald.	M	II	32	32	32	<1
Anti-N Animal Ortho 6, 9	N	III	8	4	2–4	<1
Anti-N Animal Ortho 6, 9	N	VII	16	4–8	8	<1
Anti-N Animal Ortho 12	N	VII	8	8	8	<1
Anti-M Human Cald.	MN	X	16–32	32	32	<1
Anti-N Animal Ortho 12	MN	X	4–8	1–2	2	<1
Anti-S Human saline Fa 2-214	S	VI	2	2–4	<1	1
Anti-S Human incomplete Ortho	S	VIII	4	2	<1	<1
Anti-P Human Ortho	P	VI	8–16	32	<1	<1
Anti-K Human incomplete Mor.	K	XIII	8	32	<1	<1
Anti-C Human Ortho R3080	C	X	8	32	<1	<1
Anti-C Human Ortho R3080	C	VI	4	64	<1	2
Anti-C Human Ortho R3080	C	IV	8	64	<1	<1
Anti-D Human Ortho R1208	D	IV	8–16	16	<1	2
Anti-D Human Ortho R1208	D	VI	8	4–8	±1	<1
Anti-D Human Ortho R1208	D	VII	32	32	<1	1
Anti-E Human Ortho R4056	E	XII	8–16	16	<1	<1
Anti-c Human incomplete Ortho R0035	c	VIII	8	16	<1	<1
Anti-c Human incomplete Ortho R0036	c	VIII	8	8–16	<1	<1
Anti-e Human Ortho	e	VIII	8–16	16–32	<1	1

* Expressed as reciprocal of serum dilution.

Table 1, which shows the abolishment of agglutination of virus-treated erythrocytes by anti-M and anti-N sera. Similar results were observed when an indirect anti-human globulin test was performed on red cells "sensitized" with human anti-M sera. The absorption of all blood group agglutinins other than anti-M and anti-N by virus-treated erythrocytes, which indicates unimpaired blood group agglutinogens, can be seen in the sixth column of Table 1.

Unabsorbed anti-M and anti-N sera frequently also showed a reduction in titer when tested against virus-treated erythrocytes as compared to untreated red cells (fourth and fifth columns of Table 1). This effect was observed in spite of the interference caused by the "T" antigen, which is known to be unmasked by influenza virus and R.D.E.[4] Absorption of sera with virus-treated red cells removed "T" agglutinin.

The presence of other blood group antigens had no demonstrable effect upon the

inactivation of the erythrocyte antigens M and N by influenza viruses. Erythrocytes of different genetic origin behaved identically in this respect. Unabsorbed sera containing agglutinins against blood group antigens other than M and N sometimes gave higher, but never lower, titers with virus-treated erythrocytes when compared to intact red cells (fourth and fifth columns, Table 1). This is further evidence that none of the tested blood group antigens besides M and N is impaired by these influenza viruses.

The serological findings were essentially the same when R.D.E. was used instead of influenza viruses, in that there was inactivation of M and N receptors and no demonstrable effect on any of the other blood group antigens which were examined (Table 2).

No alteration in the blood group agglutinogens studied were observed when erythrocytes were incubated in saline or in uninoculated allantoic fluid. The activity of influenza virus and R.D.E. was destroyed by boiling.

Material released by action of influenza viruses or R.D.E. upon MM or NN erythrocytes failed to inhibit, in concentrations of 10 mg and less/ml, the action of anti-M and anti-N agglutinins upon their corresponding agglutinogens.

Color reactions performed on the enzymatically released dialyzable and nondialyzable materials were positive for carbohydrates and characteristic of neuraminic acid or its derivatives. This is in agreement with our earlier observations on digests from beef and sheep erythrocytes[1] obtained under similar experimental conditions.

DISCUSSION

The fact that influenza viruses and R.D.E., which are known to elute virtually completely from human erythrocytes, inactivate blood group specific agglutinogens of the human red cell surface may throw light on the mode of action of influenza virus upon human cells. Under appropriate conditions, M and N receptors on the red cell surface appear to be destroyed. According to Kosjakov and Tribulev, the M and N factors occur in various human tissues besides the erythrocytes.[5] These observations have been confirmed by Boorman and Dodd.[6] Epithelial structures were found to contain more M and N active material than did tissue of mesodermal origin. Since all humans possess M or N agglutinogen or both, it seems possible that the blood group antigens M and N may serve as a point of attachment of influenza virus to human cells other than erythrocytes.

It is remarkable that no other blood group agglutinogen studied was affected by influenza virus or R.D.E. It was not possible, however, because of lack of specific antisera, to study the effect of influenza virus and R.D.E. on some rare blood group factors known to occur on human erythrocytes.

Attention should be drawn to the failure to find inactivation of the antigen S, because this antigen is considered to be the product of a gene very closely linked to the allelomorphic genes M and N.[7]

The specific removal of blood group M and N activity from the erythrocyte surface by influenza virus may point to the chemical nature of the M and N substances and the way in which these serologically active substances are bound to red cells because influenza virus is believed to possess only one enzyme, neuraminidase.[8] It is of interest in this respect, however, that Morton and Pickles[9] and Rosenfield and

Vogel[10] have described the destruction of M and N factors in addition to other blood group antigens by certain preparations of trypsin and by proteolytic enzyme preparations from *Streptomyces albus*. Watkins and Morgan[11] have observed simultaneous inactivation of blood group M and N antigens, one blood group antigen of the ABO(H) system, and influenza virus receptors by enzyme mixtures obtained from a protozoön, *Trichomonas foetus*. Similar findings have been reported by Howe, MacLennan, Mandl, and Kabat[12] with enzyme preparations from a bacterium, *Clostridium tertium*.

Blood group M and N antigens may be related chemically to the heterogenetic mononucleosis receptor of sheep and beef erythrocytes because this receptor is removed also by the agents found active against the M and N antigens.[1] Failure to observe any inhibition of anti-M and anti-N agglutinins by dialyzable and nondialyzable products from M and N erythrocytes is in contrast to our earlier observations on mononucleosis receptor, which, at least in part, was removed from erythrocytes but was not entirely destroyed by enzymatically active agents.[1] However, a more thorough study of optimal conditions should be made before concluding that the effect of influenza viruses and R.D.E. on M and N agglutinogens differs fundamentally from their action on mononucleosis receptor.

Interference caused by "T" agglutinin in those cases where the sera were not absorbed prior to testing was not always significant, especially when the original titer of the serum was high, e.g., Ortho anti-M R8064 and T2015, and the agglutinins anti-A and anti-B. In general, "T" agglutinins, even when present in high titer, were removed by one absorption with virus- or R.D.E.-treated red cells.

The titration of the specific blood group M and N activity of red cells before and after incubation with materials suspected of containing influenza virus may possibly be used to determine the presence or absence of these viruses in a given material and also of measuring antibodies to them.

SUMMARY

Influenza viruses and R.D.E. are capable of inactivating or removing blood group agglutinogens M and N from human erythrocytes. None of the other agglutinogens of human erythrocytes which were studied was demonstrably influenced by these agents. Inhibition tests performed with enzymatic digests indicated that the M and N agglutinogens are destroyed.

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*AMINO ACID INCORPORATION IN PIGEON PANCREAS FRACTIONS**

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The function of the pancreas is predominantly that of synthesis of proteins. This tissue has therefore attracted the attention of various workers interested in this problem, for example, Mirsky,[1] Hokin,[2] Straub.[3] However, those who have attempted to obtain cell-free preparations, either for protein synthesis proper or even for an amino acid incorporation, have encountered difficulties. Yet, as a protein-synthesizing system, pancreas holds considerable promise, and for this reason we embarked some years ago on a plan to explore, if possible, the path of protein synthesis in preparations obtained from this organ.[4, 5]

At first, we also encountered difficulties in getting not only net synthesis but also amino acid incorporation, and as a preliminary we concentrated on the characterization of amino acid-activating enzymes of the type first demonstrated by Hoagland in the liver,[6] which we found rather concentrated in pancreas extracts. The trytophan-activating enzyme was particularly abundant in beef pancreas, and a fraction was obtained from particle-free extracts of this organ representing 70 per cent pure enzyme protein.[4] Studies with this enzyme have given more detailed information on the character and mechanism of action of this type of enzyme.[5]

Meanwhile, although unable to obtain in vitro enzyme synthesis in cell-free pancreas preparations, we turned, as a more hopeful approach, to amino acid incorporation. After considerable trial and error, it was found that the pancreas of young pigeons, 6–8 weeks of age, gave homogenates that consistently incorporated amino acid into protein. At first, the pancreas system appeared to be quite similar to the liver system of Zamecnik and Keller;[7] by recombining the supernate and microsome fractions, incorporation was obtained, while microsomes and supernate alone showed only slight activity. It then appeared, however, that the supernate fraction behaved differently in the pancreas from that of the liver, since reactivation could be obtained with heated supernate, while, in the liver, heating of the supernate destroyed activity. The heat-stable factor in the pancreas was then identified as a compound similar to the polynucleotide fraction of liver recently described by Hoagland, Zamecnik, and Stephenson.[8] The pancreas system therefore appeared suited to characterize this component, and it is on attempts to do this that we want to report here.