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Antibody analysis of the parainfluenza-mumps-NDV group of myxoviruses

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PARAINFLUENZA - MUMPS - NDV
GROUP OF MYXOVIRUSES

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ANTIBODY ANALYSIS OF THE PARAINFLUENZA-MUMPS-NDV

GROUP OF MYXOVIRUSES

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INTRODUCTION

Extensive cross reactivity among the parainfluenza-
mumps-NDV group of myxoviruses has been observed in man and
experimental animals. These observations have **led** to great
confusion concerning the relationships between these viruses,
since variation is seen in different individuals and in
different animal species. The observation of heterologous
antibody production against members of this group other than
the virus shown by isolation to be the infective agent has
led to misgivings concerning the serologic diagnosis of
infection with these agents. A still unanswered question
is whether heterologous antibody represents de novo production
of that antibody in an individual, or is an anamnestic response
seen only in individuals with previous experience with the
virus to which heterologous response is evoked. The question
arises as to whether these viruses share common structural
antigenic components, as is the case with the influenza viruses.

In the present study experiments were conducted to
characterize the primary, secondary and heterologous antibody
responses with regard to 19S and 7S antibody production in
monkeys inoculated with mumps and/or DA virus. In addition,
absorption of sera with viral antigens was carried out to
determine if heterologous antibody would react with the
antigen which had caused its increased production.

Elucidation of the nature of heterologous antibody
response is important for understanding the basic relation-

ships of members of this group.. Furthermore, the understanding of these interrelationships is necessary for evaluation of serologic response in clinical diagnostic and epidemiologic studies. The possible role of the heterologous response as protective mechanism against viral infection is worthy of consideration.

REVIEW OF LITERATURE

General Consideration of Myxoviruses with Special Reference
to the Parainfluenza-Mumps-NDV Group

The name myxovirus was suggested in 1956 to classify
those viruses which could be related to Influenza A virus on
the basis of physical, chemical and biological properties.[3]
The criteria for inclusion in this group are: 1) ability to
hemagglutinate fowl erythrocytes, 2) presence of viral receptor
destroying enzyme, 3) removal of erythrocyte receptors by
receptor-destroying enzyme of vibrio cholerae filtrate, 4)
removal of "normal inhibitor" from serum by receptor-destroying
enzyme, 5) growth in amnionic cavity of chick embryo, 6) size
of 80-150 mu , 7) sensitive to ether, 8) stable when stored at
-70°C, 9) RNA virus. In addition to the influenza viruses,
mumps virus and Newcastle Disease virus (NDV), several other
viruses which meet the criteria of myxoviruses have been
isolated from man and animals; these are grouped together as
the parainfluenza viruses.

The parainfluenza viruses have been isolated from many
geographic areas, from several host species, and have been
found associated with a variety of clinical states. These
viruses were originally named for place of isolation, various
biological properties, host species or individual, or associated
clinical syndrome. This lead to a diverse nomenclature for a
group of related viruses, some strains of which seemed identical.
Andrewes et al. suggested name parainfluenza and proposed
those viruses which would make up the first three members of

this group.[4] Accordingly, parainfluenza-1 includes Sendai
(hemagglutinating virus of Japan) and hemadsorption type-2
(HA-2) viruses, parainfluenza-2 includes croup-associated (CA)
virus, and parainfluenza-3 includes hemadsorption type-1 (HA-1)
virus. Parainfluenza-4 was isolated in 1959 from adults and
children with respiratory illnesses.[32]

The name parainfluenza-5 has been proposed for a group of
viruses,[23] commonly found in captive simian populations and recently
in humans, including DA virus and SA virus.[24,45] The original
human isolation of DA virus was accomplished by Hsiung et al.
from a patient with infectious hepatitis.[24] It was demonstrated
that this myxovirus was antigenically indistinguishable from
SV-5 found in monkeys.

Studies have shown many cross reactions between different
parainfluenza viruses. These viruses have also been shown to
cross react with mumps virus and NDV. As will be enumerated
below the cross reactions occurring among these viruses are
far from consistent, varying from species to species and from
individual to individual. The close relationship of mumps
and NDV to the parainfluenza viruses was recognized by Andrewes
et al., but because of wide acceptance of the existing names
for these viruses they were not considered as parainfluenza
viruses.[4] Furthermore, clinically these two viruses are not
associated with respiratory illnesses which are characteristic
of symptomatic infection with the parainfluenza viruses.
Numerous studies have failed to demonstrate any cross reactivity
between any of the strains of influenza and the parainfluenza,

mumps or Newcastle Disease viruses.[7,10,14,30,40]   It therefore

seems reasonable to consider parainfluenza-mumps-NDV to be a

group of related myxoviruses, distinct from the influenza

viruses.

Distribution of Parainfluenza Antibodies in Human Populations

Several serological surveys of populations from around the

world have indicated that these viruses are ubiquitous and that

the incidence of antibodies to each virus is similar in the

different areas studied. LaPlaca and Mosovivi studied a large

number of sera from India, Italy and Colorado. They found

86-97.5% positive reactors to parainfluenza-3, 60-80% to

parainfluenza-1 and 8-22% to parainfluenza-2.[39] In New Haven

a study of 304 hospitalized patients showed the following

incidences of positive serologic reactions: parainfluenza-1,

54%; parainfluenza-2, 63%; parainfluenza-3, 83%; DA virus,

74%; and mumps, 70%.[26] Chanock found 90% of young adult males

he tested to have antibody against parainfluenza-2.[7] A study

of residents of southern Louisiana showed prevalence of

antibodies against these viruses to be: 75% for parainfluenza-3,

37% for parainfluenza-2, 19% for HA-2 and 9% for Sendai virus.[19]

Clinical States Associated with Parainfluenza-Mumps-NDV Infections in Man

The parainfluenza viruses have been most commonly isolated

from children and adults with respiratory illnesses. The most

common symptoms are rhinnorhea, fever and cough, occassionally

symptoms of pneumonia are seen.[43] The most serious condition

definitely known to be caused by these agents is viral croup

in infants and young children.[8,10,21,52] These agents,
particularly parainfluenza-1, are the most common cause of
croup in children under 2 years old. The clinical syndrome
of parotitis caused by mumps is well known. Newcastle Disease
virus infection has rarely been documented in humans, and then
only in association with mild conjunctivitis.[18,35] The serological
studies cited above would indicate all of these viruses, except
NDV, are associated with high incidence of subclinical infection.
In a study by Horstmann and Hsiung, parainfluenza-2 virus was
isolated from three cases of sudden unexplained death of infants.[21,52]
This suggests that these viruses may be associated with serious
sequelae in infants.

Parainfluenza Virus Infections in Animal Populations

The viruses of the parainfluenza-mumps-NDV group are also
widespread among domestic animal populations. Parainfluenza-3
is the etiologic agent for "shipping fever" in cattle, a
respiratory illness manifested only when cattle are crowded.[1,20,37]
Seventy per cent of cattle slaughtered in the United States have
antibody to parainfluenza-3.[2] Parainfluenza-3 has recently been
isolated from fatal cases of pneumonia occurring in patas monkeys
crowded after transportation from Africa to England.[12] The
monkeys were shown to acquire parainfluenza-3 after captivity,
and the virus isolated was similar to that found in humans.
Hsiung has reported 79% of monkeys arriving in New Haven to
have parainfluenza-3 antibodies.[23] DA virus is very common in
captive monkeys, but antibody against this virus which is
identical to the simian virus, SV-5, is rare in wild monkeys.[24]

This evidence suggests that parainfluenza-3 and -5 may be acquired by susceptible animals after they are brought into contact with human environment. Incidence of antibody against these viruses in laboratory animals received in New Haven was determined by Hsiung et al. Monkeys, rabbits and guinea pigs had high incidence of antibody against all members of this group except NDV, hamsters had only DA antibodies, while mice and chickens had none.[23] It is important to keep these data in mind when evaluation experiments utilizing laboratory animals.

Cross Reactivity Among the Parainfluenza-Mumps-NDV Group

Cross Reactivity in Man: In 1956, Chanock isolated CA virus (parainfluenza-2) from infants with croup. At this time he noted that four of eight patients with mumps showed a heterologous rise in HI anitbody to this newly isolated myxovirus.[7] Subsequently, Chanock et al. studied two separate acute episodes of febrile illness in nursery schools, in one parainfluenza-1 was shown to be the etiologic agent and in the other parainfluenza-3. Heterologous antibody rise was seen in both populations to the non-infecting one of these two viruses with 33% involved in the parainfluenza-1 outbreak having parainfluenza-3 rise, whereas 12% of the other group showed heterologous rise. Most of those with heterologous response had had pre-existing antibodies. The viruses which were isolated produced no heterologous response in hamsters without pre-existing antibody.[11] The greater incidence of heterologous parainfluenza-3 antibody was believed due to greater previous experience with this

virus. An earlier study by Chanock of children with respiratory illnesses found no heterologous rise to parainfluenza-1 or -2 or mumps and only 1 in 10 with parainfluenza-1 had a rise to parainfluenza-3.[8]

The patient in whom the original isolation of parainfluenza-4 was made showed heterologous response to parainfluenza-3, but not parainfluenza-1 or -2. It has been shown that adults, but not children, have heterologous response to mumps when infected with parainfluenza-4.[32]

Cross reactivity with parainfluenza-5 in humans is poorly understood. Chanock concluded the low number of individuals with CF antibody to SV-5 had this as a result of infection with parainfluenza-2.[9] He concluded, that there is no evidence for human infection caused by this agent. Evidence is cited above that DA virus indistinguishable from SV-5 can cause human infection.[25]

The antigenic relationship between mumps and NDV has been observed since 1949.[34,36] In this case previous experience with NDV is presumed to be unnecessary for the commonly observed NDV antibody found in patients convalescent from mumps, and it is believed naturally occurring NDV antibody in the population is the result of mumps infections.[33] The close relationship of these two viruses is borne out by common biological properties of hemolysin activity[6] and their proximity on the erythrocyte receptor gradient.[42]

DeMeio and Walker found 22 of 24 young adult patients with clinical mumps to have heterologous antibody rise to

parainfluenza-1. They also found positive correlation between antibodies to parainfluenza-1 and mumps in sera from well medical students. From these findings they concluded, "It is quite likely that much, and perhaps most, of the antibody against HVJ (parainfluenza-1) found in the general population of the United States is attributable to mumps virus infection rather than actual experience with HVJ."[16] In the years since this study, parainfluenza-1 has been shown to be a common respiratory pathogen in the United States.[11,21] Heterologous responses to parainfluenza-2 and -3 have also been noted in mumps patients.[7] Lennette et al. studied sera from 47 mumps patients and found 40% heterologous rise to parainfluenza-1, 21% to parainfluenza-3, and 8% to parainfluenza-2. They found the incidence of heterologous response to increase with age.[40] These workers along with others previously cited propose that in order for a heterologous antibody response to occur among the parainfluenza and mumps viruses, pre-existing antibodies must be present. It is of note that in the instances cited,[9,16] demonstration of cross reactivity lead to the false conclusion that parainfluenza-1 and -5 were not a frequent cause of human infection in certain populations with antibodies against these agents.

Hsiung et al. studied antibody response in 10 patients in whom parainfluenza viruses were isolated. Extensive cross reactions were seen in these patients. Most puzzling among these patients was a 1 month old infant who had no titer to parainfluenza-1, -2, -3, mumps or DA when parainfluenza-2 was

isolated; however, 3 months later had antibody to all of these
viruses demonstrated.[25]

Cross Reactivity in Experimental Animals: Animal experiments
have in general showed extensive cross reactivity among these
viruses similar to that seen in human studies with some
differences according to the species used. Chanock et al.
showed that guinea pigs which have parainfluenza-3 antibodies
will have an increase in this antibody when inoculated with
mumps or parainfluenza-1, whereas all animals without pre-
existing parainfluenza-3 antibodies and some with them failed
to show this heterologous response.[8] Cook et al. found
individual guinea pigs to show different heterologous responses
which indicated relationships between: parainfluenza-1 and -2;
parainfluenza-1 and -3; parainfluenza-3 and mumps.[14] Using
chicken and guinea pig immune sera prepared against parainfluenza-1,
mumps and NDV, Ishida et al. showed cross reactivity between
these viruses.[27] Similar relationships between these viruses
have been shown using rabbit immune sera.[53] Hamsters inoculated
with parainfluenza-3 showed no anitbody rise to parainfluenza-1
or -2, or mumps.[15]

Heterologous antibody responses to inoculation with mumps
and DA virus in different species of laboratory animals was
carried out by Hsiung. Only animals without antibodies to
mumps and DA were used. Rabbits developed mumps antibody
after DA inoculation, but only showed specific response to mumps
inoculation. Guinea pigs showed heterologous response to both
viruses, whereas hamsters showed only specific antibody rise.

Chickens had heterologous rise to parainfluenza-3 and NDV
(they had previous inoculation with NDV) after mumps or DA.
Monkeys inoculated with DA virus showed heterologous response
to mumps and parainfluenza-3, and rarely to parainfluenza-1 or
-2.   Hsiung considered the possibility that the animals had
previous contact with the viruses to which they showed a
heterologous rise since these responses were seen in those
species which had high incidence of natural infection.[23]

Studies of Antibody Structure

Antigen-Antibody Absorption: Absorption of antibodies by viral
particles is one method of demonstrating the presence of
structural antigenic components shared by more than one virus.
A known specific viral antigen is added to serum, antigen-
antibody complexes are allowed to form, and the virus particles
(with any attached antibody) are removed from the mixture. Serum
is tested for antibody against those viruses in which common
components are suspected before and after the absorption
procedure. A decrease in titer of antibody against a virus
other than that used for the absorption is evidence of common
antigen shared by these two viruses. Removal of viral particles
from the serum mixture is accomplished by ultracentrifugation
or absorption onto erythrocytes, the latter being limited to
those viruses which exhibit hemagglutination.

Using antibody absorption techniques, Jensen and Francis
have determined that 42 strains of Influenza A share 18 antigenic
components.[29] Clarke has shown common antigens shared by Group B

arborviruses.[13]

In the only published report of absorption analysis of the parainfluenza-mumps-NDV group, Hsiung found only homologous antibody was removed by DA virus and parainfluenza-3 antigen. In this study sera was used from a chimpanzee which had been inoculated with DA virus had shown an antibody rise to DA, parainfluenza-1 and -3, and mumps.[23] Jensen et al. have shown that certain strains of influenza virus will not absorb antibody to parainfluenza-1.[28]

Determination of 19S and 7S Components: In recent years it has been noted that two types of antibody, differing in molecular size, are formed in response to antigenic stimulus. A macroglobulin with sedimentation coefficient of 19 Sverberg units (19S) appears in the initial phase of a primary response. Later smaller globulin, 7S, appears and persists for long periods of time.[5] This smaller antibody seems to be the important measurable factor in the "immunological memory system." A secondary response is characterized by rapid production of large amounts of 7S antibody, formerly thought without production of 19S. Svehag and Mandel have shown that in rabbits inoculated with polio-virus about 50 times as much antigen is required to cause 7S production as 19S production, and that if doses of antigen insufficient to induce 7S production are used a secondary response on reinoculation will not occur.[48] Further they have shown that the secondary response, in their test system, is characterized by a production of 19S antibody similar to that seen in the primary response.[49] The 7S antibody

responded to secondary stimulus with a rapid 200-fold increase

so that 19S, present in much smaller quantities, could only be

detected when separated from 7S by ultracentrifugation. Svehag

and Mandel also cite evidence that cessation of 19S, 4-5 days

after inoculation appears due to loss of antigenic stimulus

which is necessary for continued 19S production, but not 7S

production once it is initiated.[49] They postulate two different

cell populations produce these different types of antibody. The

conclusions of Svehag and Mandel are in aggreement with those

of Uhr and Finkelstein who studied the immunologic response

of rabbits inoculated with bacteriophage.[50]

Separation of 19S and 7S is accomplished by differential

centrifugation across a density gradient of sucrose or cesium

chloride.[5] It has been found that 2-mecaptoethanol (2-ME)

will destroy activity of 19S but not 7S antibody.[5] This has

been shown to occur by breaking disulfide bonds in the

macroglobulins.[17] Treatment of serum with 2-ME is a useful

way of determining presence of 19S antibody; however, it will

show decrease of antibody titer only if 19S makes up a large

part of the antibody present. The relative insensitivity of

this technique accounts for the conclusion reached by some that

there is no production of 19S in a secondary antibody response.[41,51]

MATERIALS AND METHODS

Virus Strains

Mumps. Two strains of mumps virus were used. One strain
(DD_4) was obtained from Dr. Werner Henle who had isolated it from
a patient in 1954. Isolation was in Hela cell tissue culture,
and it was passed four times before storage at $-70^{\circ}C$. Before
use in these experiments the virus was passed six times by
amniotic inoculation of 6 day old chick embryos, the fluid
being harvested after 7 days. The fluid contained 10^7 EID per
ml. The second strain used was Enders strain mumps virus.

DA Virus. Tissue culture fluid from monkey kidney tissue
cultures infected with DA virus (Rh-327)*was used. The fluid
contained $10^{6.5}$ TCID per ml.

Parainfluenza-3. Parainfluenza-3 (Rh-10)*was grown in
monkey kidney tissue culture in the presence of SV-5 antisera.
The fluid contained $10^{3.5}$ TCID per ml.

Animal Inoculations

Monkeys #1-4. Four rhesus monkeys were inoculated with
mumps virus, numbers 1 and 3 via the parotid duct as described
by Johnson and Goodpasture[31] and numbers 2 and 4 by instillation
of the inoculum into the nasopharynx. One ml. of inoculum
containing 10^7 EID of DD_4 strain mumps virus was given to each
animal.

After inoculation the animals were watched daily for signs
of illness. There were no changes in temperature, parotid size
or consistancy, or in eating pattern to indicate illness.

* Number of passages in rhesus tissue culture

Attempts to isolate mumps virus from saliva and urine were carried out. Saliva was collected 2, 4, 6, 8, 11 and 14 days after inoculation and urine on days 6 and 14. The saliva was collected with a sterile cotton swab which was pulverized in 1.5 ml. of Hanks balanced salt solution containing 150 mg. streptomycin and 150 units penicillin. One-tenth ml. of urine was put into 1.5 ml. of the same Hanks solution. These samples were cleared by centrifugation. Of each sample in Hank's solution 0.1 ml. was inoculated into each of three 6 day old chick embryos and 0.2 ml. into each of three 3 day old chick embryo fibroblast cultures. The egg fluids were harvested after 5 days and checked for hemagglutination of 0.5% chick erythrocytes. The fluids from each group of three eggs were pooled and inoculated into 3 more chick embryos and hemagglutination looked for after 5 more days. The chick embryo fibroblast cultures were checked for hemadsorption of guinea pig erythrocytes at 3, 5, 7 and 10 days.[43] All attempts at isolation of mumps virus were negative.

Blood for serologic study was obtained from each animal previous to inoculation and 4, 6, 8, 11, 14, 19, 22, 30, 57 and 90 days after inoculation. Serum was separated from clot and stored at -20°C until used for antibody determination.

Ninety days after the first inoculation with mumps virus, inoculation of these animals with DA or parainfluenza-3 virus was carried out. Monkeys #1 and 2 were given inoculation with DA virus. One ml. of monkey kidney tissue culture fluid containing $10^{6.5}$ TCID per ml. was given intravenously and 1 ml.

intranasally to each monkey. Monkeys #3 and 4 received similar amounts of tissue culture fluid containing $10^{3.5}$ TCID of parainfluenza-3 virus. Blood was collected from each animal at 5, 8, 14, 21 and 30 days after inoculation.

Monkeys #2 and 3 were given a second inoculation with mumps virus 6 months after the first and 3 months after the inoculation with DA in case of #2 and parainfluenza-3 in case of #3 (Table 1). The virus used was the same as that used for the first inoculation having been stored at -70°C. One ml. was given to each monkey intravenously and 1 ml. intranasally. Blood was collected before and 2, 4, 8, 11, 15, 22 and 30 days after inoculation.

Inoculation of Monkeys #5-7. Three monkeys which had previously been inoculated with different viruses were selected for mumps inoculation to study the response of animals with known histories of infection. Monkey #5 had been inoculated with parainfluenza-1 five months previously; monkey #6 with polio-1 five months previously; and monkey #7 had received DA virus ten months and parainfluenza-1 eight months previously (Table 1). Inoculation with mumps was carried out by intravenous and intramuscular injection of 10^{7} EID of Enders strain mumps virus per animal (Table 1). Serum was collected before and 2, 4, 7, 14, 21 and 30 days after inoculation. No signs of illness occurred in these animals. Isolation of virus was not attempted.

Chimpanzee Inoculation with DA Virus. Stored sera from chimpanzee (Mack #50) in which DA virus had been inoculated was furnished by Dr. Hsiung to provide sera representing a

primary response to DA virus inoculation. This animal which had no pre-existing DA antibodies had been inoculated with DA virus intravenously.

Antibody Determinations

Hemagglutination-inhibition (HI) titers were determined using the viral antigens of the parainfluenza-mumps-NDV group. The complement fixation test was not used because it has been found unsuitable for evaluation of activity against DA virus due to anticomplementary effect when commercial preparations of complement is used with DA antigen.[25] Studies in humans have shown HI and neutralization tests to be more specific than CF for these viruses.[11] Hemagglutination-inhibition tests were chosen as it was desired to test large numbers of sera against several antigens which could be done with one test system; whereas, both tissue cultures and embryonated eggs would be required to do neutralization tests against all the viruses concerned.

Hemagglutination-Inhibition Test.

1. Serum Treatment. Sera were heat inactivated at 56°C for 30 minutes. Serum agglutinins were removed by adding packed washed guinea pig erythrocytes to serum diluted 1:5, keeping the mixture in ice bath for 30 minutes, and then removing the erythrocytes by centrifugation. An equal volume of 25% kaolin in PBS was added to the 1:5 dilution of serum to remove nonspecific serum inhibitors. After 20 minutes at room temperature the kaolin was removed by centrifugation yielding a 1:10 dilution of serum.[22]

2. <u>Antigen Preparation</u>. Mumps viral antigen
obtained from Lederle (Lot number: 2540-36); Parainfluenza-3
and DA were grown in rhesus monkey kidney tissue cultures and
the fluids used for hemagglutinating antigens. Parainfluenza-3
was grown in presence of SV-5 antisera.

3. <u>Preparation of 0.5% Guinea Pig Erythrocyte Suspension</u>.
Guinea pig erythrocytes were washed three times by suspension
in PBS and centrifugation. After the third washing the packed
cells were suspended in approximately an equal volume of PBS.
The exact percentage of erythrocytes in this suspension was
determined by use of capillary hematocrit tubes which were
centrifuged in an International microcapillary centrifuge. The
cell suspension was appropriately diluted to produce a 0.5%
suspension in PBS.

4. <u>Test Procedure</u>. Two-fold dilutions of the treated
serum in 0.2 ml. amounts were made with PBS in plastic cup
panels by use of a serologic diluter. Two-tenths ml. of
antigen containing 4 hemagglutinating units was added to each
cup. After one hour at room temperature, 0.4 ml. of the
erythrocyte suspension was added. The panels were examined
for hemagglutination after two hours. The HI titer was recorded
as the reciprocal of the highest serum dilution showing no
hemagglutination.

<u>Analysis of Antibody Components</u>

<u>Treatment of Sera with Mecaptoethanol</u>. Serum was heat
inactivated, serum agglutinins removed with guinea pig

erythrocytes and diluted 1:5 with PBS. To 0.9 ml. of this treated serum 0.1 ml. of 1 molar mecaptoethanol (2-ME) in PBS was added; 0.1 ml. PBS was added to control sera. The serum was incubated overnight at 4°C to allow inactivation of 19S antibody. After incubation the serum was treated with kaolin and HI tests performed.

Centrifugation of Sera Across Sucrose Gradient. Sucrose gradients were prepared in 5 ml. cellulose nitrate ultracentrifuge tubes. The first method used was to layer 0.9 ml. amounts of sucrose solutions ranging from 37% on the bottom to 12.5% on top. This produced a layered symmetric gradient.[44] The second type of gradient used was an asymmetric sucrose gradient prepared by drop-wise addition of 40% sucrose to a 10% solution. The asymmetric gradients were prepared with the apparatus and method of Speir.[47] Two-tenths ml. of serum, which had been treated with guinea pig erythrocytes to remove serum agglutinins, was layered over the gradient. The gradients were centrifuged for 16.5 hours at 35,000 RPM in a Spinco Model L ultracentrifuge with a SW39 centrifuge head. After centrifugation, fractions of the gradient were collected in drops from a needle hole in bottom of the tube. Nine fractions of 0.5 ml. were collected from each tube. Each fraction was diluted 1:2 with PBS. To 0.5 ml. of each diluted fraction 0.05 ml. of 1 molar 2-ME was added; 0.05 ml. of PBS was added to the remaining 0.5 ml. The fractions were kept overnight at 4°C before doing antibody determinations.

Absorption of Antibody with Viral Antigens

Antigen Preparations. 1. Mumps--viral antigen obtained

from Lederle (Lot number: 2540-36) which had hemagglutination
titer of 1:1280 was used.

2. DA virus--tissue culture fluid from monkey kidney cell
tissues infected with DA virus was used for antigen source. To
1000 ml. of the fluid 20 ml. of packed guinea pig erythrocytes
was added. Adsorption of virus onto the erythrocytes took place
in 4 hours at room temperature. The mixture was centrifuged at
1500 RPM for 15 minutes and the supernatant fluid removed. To
the guinea pig erythrocytes which had virus attached, 50 ml. of
PBS was added. This mixture was kept in 37°C water bath for 1 hour
with intermittent shaking. After the elution of virus the erythrocytes
were removed by centrifugation and the supernatant used for antigen.
The final antigen solution had hemagglutination titer of 1:1280.

3. Parainfluenza-3--antigen was prepared from infected
monkey kidney tissue culture by erythrocyte adsorption-elution
as described for DA virus. The antigen preparation had
hemagglutination titer of 1;640.

4. Newcastle Disease virus--infected egg fluid with
hemagglutination titer of 1:640 was used (California strain 11914).

Absorption of Sera. Equal volumes of 1:10 dilution of serum
treated as described above in preparation for HI tests and the
antigen suspension were mixed and kept overnight at 4°C. The
virus-antibody aggreagates were removed by ultracentrifugation at
40,000 RPM for 4 hours in Spinco Model L ultracentrifuge with No. 40
centrifuge head. The supernatant fluid was checked for
hemagglutination and treated with guinea pig cells when
necessary to remove agglutinins. To control sera PBS was added
in place of antigen.

RESULTS

Antibody Response to First Mumps Inoculation

Monkeys #1-4.(Figures 1-4) Inoculation of mumps virus
into monkeys #1-4 caused production of mumps HI antibody which
reached maximal levels 19 to 22 days after inoculation in all
four animals. The antibody levels were first detectible
between 4 and 8 days in low titer. This low level of initial
antibody production is characteristic of primary response to
a viral infection. Since these animals were inoculated via
parotid duct or nasopharynx it was assumed that this antibody
response indicated an infection occurred in spite of failure
to isolate mumps virus. The titers of mumps HI antibody
decreased after 22 to 30 days, but were still significant at
90 days after inoculation.

Only monkey #1 showed a heterologous antibody response to
DA virus after mumps inoculation. As shown on Figure 1, this
response closely paralleled that of the response to mumps. All
four animals had initial DA virus HI titers of 40 to 80, yet
only one showed a heterologous response. None of the animals
responded with a rise of parainfluenza-3 HI antibody. High
levels of antibody against parainfluenza-3 were present at the
time of mumps inoculation.

Monkeys #5-7. (Figures 5-7) These monkeys which were
inoculated by intravenous and intramuscular injection of Enders
strain of mumps virus responded with a rise of mumps antibody
less pronounced than that seen in monkeys #1-4. Monkey #6
had initial mumps HI titer of 40 while the other two had titers

of 10. The presence of pre-existing antibodies indicates the
probability in one, and perhaps all three, of previous
experience with mumps virus.

None of the animals showed heterologous response with
antibodies to DA virus or parainfluenza-3 to which they had
significant pre-existing HI antibody titers.

Antibody Response to DA Inoculation

Monkeys #1 and 2. (Figures 1 & 2) Inoculation of DA
virus was followed by a rise in DA virus HI antibodies in
both animals. This rise was marked at 5 days and reached
maximal level at 8 days. The rapidity and magnitude of this
response are characteristic of a secondary, or anamnestic,
antibody response.

Both monkeys showed an eight-fold heterologous rise in
HI antibody to mumps after DA inoculation. This response was
of less magnitude than that seen after the first mumps inoculation,
but was of similar temporal progression. Monkey #1 showed a
four-fold increase in HI antibody to parainfluenza-3 after
DA virus.

Antibody Response to Parainfluenza-3 Inoculation

Monkeys #3 and 4. (Figures 3 & 4) Monkey #3 which had HI
titer of 1280 against parainfluenza-3 at the time of inoculation
showed no significant response to this virus. Monkey #4 which
had initial titer of 160 showed a rapid response characteristic
of an anamnestic rise.

A four-fold increase in DA titer was seen in monkey #3,

but no significant heterologous response in monkey #4.

Neither animal showed a heterologous response against mumps.

Antibody Response to Second Mumps Inoculation

Monkeys #2 and 3. (Figures 2 & 3) Both monkeys showed an increase in mumps HI titer of more than 100-fold by eight days. At the time of this inoculation, both animals had low levels of mumps HI antibody which had persisted since first mumps inoculation six months previously.

Neither animal showed an increase in DA virus HI antibody titers. The sera from these monkeys were also tested for antibodies against parainfluenza-2 and NDV; no antibodies to these viruses were found.

Sensitivity of HI Antibodies to 2-ME Treatment

The antibody titers of sera after treatment with 2-ME are shown on Figures 1-7. A two-fold or greater decrease in HI antibody titer against mumps is seen in all sera tested from monkeys #1-4 during the first fourteen days after inoculation (Figures 1-4). This is further evidence that these animals experienced a primary infection with mumps. A two to four-fold decrease after 2-ME treatment is seen in sera obtained 2, 4 and 7 days after mumps inoculation.from monkeys #5 and 7, but not from #6 which had higher pre-existing antibodies and less response to inoculation. A decrease in mumps titer with 2-ME treatment is also seen in sera from monkeys #1 and 2 taken during the period of heterologous antibody rise following DA inoculation (Figures 1 & 2). The titer of mumps

antibody following a second inoculation with mumps was not decreased by 2-ME treatment (Figure 2).

As shown in Figures 1-7, DA virus and parainfluenza-3 antibody titers were found not to be affected by 2-ME treatment in all these sera tested. None of these sera represented a primary inoculation, although both homologous and heterologous rises were seen in the groups tested. Sera from chimpanzee #50 which had an HI titer of 20 three days after primary DA inoculation was treated with 2-ME; antibody activity could no longer be detected at 1 to 10 dilution (not shown in figures).

Results of Separation of 19S and 7S Antibodies by Sucrose Gradient Centrifugation

Because of the inability of 2-ME treatment to detect 19S antibody in the presence of greater amounts of 7S antibody, certain sera were centrifuged across sucrose gradients to separate 19S from 7S antibodies. The results of centrifugation are shown in Figures 8-16. The hemagglutination-inhibiting activity of each fraction is indicated as the reciprocal of the highest dilution of the fraction giving complete inhibition in the system described for antibody determination. This HI activity represents the HI antibody present in each fraction, but does not represent the actual serum antibody titer because the dilution in each fraction depends upon the sedimentation properties of the antibodies. The recorded titers indicate the relative amount of HI antibody in each fraction. The findings shown in Figures 8-16 indicate that the 2-ME labile

activity--19S antibody--is limited to the lower fractions, whereas 2-ME stable activity--7S antibody-- is concentrated in the higher fractions.

Activity of serum from monkey #1 eight days after first mumps inoculation is shown in Figure 8. All mumps antibody was in the bottom fraction, whereas all DA activity is in fractions 5-7. The antibody against mumps was all destroyed by 2-ME, whereas that against DA virus was not. These findings confirm those presented above (Figure 1) that soon after mumps inoculation 19S antibody against mumps is present, but not against DA virus. In contrast, serum taken 90 days after mumps inoculation and just before DA inoculation had no 19S antibody (Figure 9).

As shown in Figure 1, monkey #1 had a rise to mumps following DA inoculation. It is also shown on Figure 1, that there was a decrease in mumps antibody, but not DA antibody, after 2-ME treatment of serum obtained 8 days after DA inoculation. The results of centrifugation of this sera obtained during the period of homologous DA rise and heterologous mumps rise are shown in Figures 10 and 11. Both figures show 2-ME labile antibody to mumps virus, but better separation of the 2-ME labile--19S--antibody and 7S antibody is seen in Figure 11 which shows the results when the asymmetric gradient was used. A two-fold decrease in activity of DA antibody was seen in the bottom three fractions with the sample centrifuged in the asymmetric gradient (Figure 11), but not that in the symmetric gradient (Figure 12).

Serum from monkey #2 obtained 14 days after first mumps

inoculation showed 19S and 7S antibody to mumps, but only 7S
to DA virus (Figure 12). Figures 13 and 14 show the results
of centrifugation of serum from monkey #2 taken 8 days after
DA inoculation. The results are similar to those for monkey #1
taken 8 days after DA inoculation. Comparing Figure 13 to
Figure 11, it can be seen that both show decrease in DA activity
after 2-ME treatment of the lower three fractions, better seen
with sera from monkey #2 than #1. In both animals tested
heterologous rise in mumps antibody after DA inoculation was
accompanied by 19S mumps antibody production.

Figures 15 and 16 show the results of centrifugation of
serum from monkey #2 during a secondary response to mumps
virus. The presence of 19S is shown by the four-fold decrease
in titer seen in fraction 1 of the asymmetric gradient after
treatment with 2-ME. The great amount of 7S present in this
sera almost obliterates the 19S peak which can be demonstrated
only in the bottom fraction of the asymmetric gradient.

By use of sucrose gradients the presence of 19S antibody
in the secondary response of monkeys to mumps and DA virus
could be demonstrated, although amounts of 19S were too small
for detection with 2-ME. Further 19S antibody against mumps
was shown to be produced in response to DA inoculation. No
19S DA antibody was seen in response to primary or secondary
mumps inoculation. The asymmetric gradients were shown in
the three instances used to be superior to the symmetric gradients
for separation of 19S and 7S activity of the serum samples used.

Antibody Absorption by Viral Antigens

Results of absorption by mumps, DA, parainfluenza-3 and NDV antigens of sera obtained from the seven monkeys and chimpanzee #50 are shown in Tables 2 and 3. The sera used represent the following antibody responses: 1) early and late primary mumps inoculation, i.e. 11, 14 and 22 days following first mumps inoculation in monkeys #1-4, 2) heterologous mumps response to DA inoculation, i.e. 8 days following DA inoculation in monkeys #1 and 2, 3) secondary mumps inoculation, i.e. 11 days after second mumps inoculation in monkeys #2 and 3, 4) primary inoculation with DA, i.e. chimpanzee #50, 5) response to DA inoculation in monkeys with pre-existing DA antibodies, i.e. monkeys #1 and 2, sera from 8 days after DA inoculation, 6) heterologous DA rise in response to mumps, i.e. monkey #1, sera from 22 days after first mumps inoculation.

In all instances specific antibody was completely removed or greatly reduced in titer by absorption with the homologous virus. Regardless of the virus used to stimulate antibody production manifest in a given serum sample, no significant decrease in titer of antibody against any virus other than that used for absorption was seen.

To rule out the possibility that failure to demonstrate cross absorption was due to insufficient amount of antigen, a second absorption procedure was carried out. This was done after the sera was shown free of antibody against the virus used for absorption. No decrease in heterologous antibody occurred with this further absorption.

DISCUSSION

Studies cited in Review of Literature of antigenic
cross reactivity between these viruses have been of two types:
1) survey of populations for antibodies against these viruses,
with or without concomitant attempts at virus isolation, and
2) studies of the immunological response of laboratory animals
inoculated with one or more of these agents. Studies of the
first type offer disadvantages that: 1) the infecting agent
cannot always be known with certainty, 2) possibility of
multiple infection cannot be eliminated, 3) previous experience
with agents is difficult to evaluate, 4) host factors cannot be
evaluated or controlled, 5) mode of infection is not controlled,
6) changes may occur in viral properties as virus undergoes
uncontrolled passage in the environment. Studies with laboratory
animals eliminate some of these disadvantages, but conclusions
from these studies must be applied to consideration of human
infections with caution.

The high rate of natural infections of captive monkeys
with parainfluenza-3 and -5 was confirmed in these studies.
Of particular note in monkeys #1-4 was the high initial titers
against parainfluenza-3. These titers decreased somewhat over
the course of the study and may indicate the animals had
acquired natural infection with this virus shortly before their
use in these experiments, a similar circumstance to that found by
Churchill.[12] The high level of parainfluenza-3 antibody makes
heterologous response to this virus difficult to evaluate.
Because all animals used had pre-existing titers to these

viruses, whether or not previous experience with one of these viruses is necessary to show a heterologous response could not be evaluated directly. It was shown that a heterologous response does not always occur even when pre-existing antibodies are present, but varies from individual to individual. Because of the widespread occurrence in laboratory animal populations of the viruses under study it is difficult to evaluate previous viral infections in the individual animals used.

Cross reactivity in terms of ability to evoke heterologous antibody responses to members of the parainfluenza-mumps-NDV group has been shown to occur. One animal in seven showed a rise in DA antibody with mumps infection; both animals inoculated with DA virus showed mumps rise. One of two animals inoculated with parainfluenza-3 responded with rise to DA. As cited earlier other workers have found extensive, inconsistant cross reactivity among these viruses.

The findings that 19S antibody is present early in primary infection and that 7S persists long after disappearance of 19S are in keeping with the work of others already cited. The presence of small amounts of 19S in secondary response to viral inoculation is in agreement with the findings of Svehag and Mandel that 19S antibody, in much smaller amounts than 7S, is found in anamnestic response.[49]

The data presented also show that 19S antibody was produced against mumps in response to DA virus inoculation. At the time DA virus was inoculated only 7S mumps antibody

was present. Other workers have postulated on the basis of
their findings that presence of antigen is necessary for 19S
production.[49,50] According to this theory 19S antibody
formation occurs only while there is persistance of antigen,
rapidly falling off after loss of antigenic stimulus. The
presence of 19S mumps antibody after DA inoculation would
indicate, according to this theory, that mumps antigen may
be present. Many viral infections, including mumps, have
been shown to persist for several months in a quiescent state.
While this cannot be definitely ruled out, absence of 19S
antibody just before DA inoculation makes it less likely. The
other possibility in keeping with this theory is that DA virus
provides the necessary antigenic stimulus for 19S mumps antibody
production. This explanation implies the presence of a common
structural antigenic component shared by these two viruses which
is important in providing a stimulus for mumps HI antibody
production.

To evaluate this possibility that the heterologous antibody
responses were due to common antigenic components shared by
members of the parainfluenza-mumps-NDV group, absorption studies
were carried out. The data presented show that HI antibody
against mumps, DA or parainfluenza-3 will not react with either
of the other two or NDV. Thus, the mumps antibody which was
caused to increase in titer by DA virus could not be absorbed
with DA virus. These studies indicate that common antigenic
components could not be the explanation for the cross reactivity
occurring between these viruses.

The evidence presented would question whether or not

persistence of antigen is necessary in all cases for
production of 19S antibody. The real significance of heter-
ologous 19S antibody cannot be stated. A heterologous rise
to DA following mumps inoculation in monkey #1 was not accompanied
by detectible 19S production.

Several workers have suggested the importance of host
factors in determining whether or not a heterologous response
will occur. The factor which has been suggested is previous
experience with the virus, a heterologous response representing
a special type of anamnestic antibody response.[11,23,32,40] The
data presented in this thesis are consistant with this theory,
but do not offer proof that the pre-existing antibodies are a
necessary factor. There must, however, also be viral factors
operative in the production of a heterologous response, for
a response against one of these agents is evoked by other
members of this group. Thus, some factor--other than shared
antigens--must be present during an infection with one of the
parainfluenza-mumps-NDV viruses which causes the individual to
react by producing heterologous antibody in certain cases. The
delineation and relative importance of these viral and host
factors awaits further investigation.

Certain points can be inferred from the data presented.
A rise in antibody against one of the agents studied may
result from infection with another one of these agents;
therefore, serologic diagnosis alone is inadequate. Demonstration
of 19S antibody alone is not proof of a primary response, as 19S
antibody may be found in a secondary response or a response

initiated by a virus other than that to which the specific 19S antibody is found. The ability of a virus to cause production of heterologous antibody does not necessarily mean it shares antigens with the heterologous virus, or that the antibody produced will react with the virus causing its production. Failure of cross reactivity of antibodies to these viruses, along with the evidence that pre-existing experience with a virus is a factor determining heterologous response, make it seem unlikely that significant cross protection is afforded by infection with one of these viruses.

SUMMARY

Cross reactivity as shown by heterologous antibody response in monkeys was demonstrated between mumps, DA and parainfluenza-3 viruses. Presence of 19S antibodies was demonstrated to mumps after primary and secondary mumps inoculation and after DA inoculation which resulted in a mumps antibody rise. After mumps inoculation, 19S antibody to DA was not present although one animal showed DA antibody rise, but 19S antibody was present after DA inoculation. Absorption of sera with mumps, DA, parainfluenza and NDV antigens showed no cross absorption of any antibody present. Thus, it was shown that cross reactivity as expressed in ability to evoke a heterologous antibody response was not associated with common antigens which could be demonstrated by absorption in the parainfluenza-mumps-NDV group of myxo-viruses.

Animal No.	First Inoculation	Second Inoculation		Third Inoculation	
	Virus	Virus	Months after first	Virus	Months after second
1	Mumps	DA	3		
2	Mumps	DA	3	Mumps	3
3	Mumps	Para-3	3	Mumps	3
4	Mumps	Para-3	3		
5	Para-1	Mumps	5		
6	Polio-1	Mumps	5		
7	DA	Para-1	2	Mumps	8

Table 1.   Sequence of virus inoculations in Rhesus monkeys.

MONKEY NO	NO. OF INOC.	VIRUS INOC.	DAYS AFTER INOC.	CONTROL			ABSORB c̄ MUMPS			ABSORB c̄ DA			ABSORB c̄ PARA-3			ABSORB c̄ NDV		
				M	DA	P-3	M	DA	P-3	M	DA	P-3	M	DA	P-3	M	DA	P-3
1	FIRST	MUMPS	11	40	40	320	<20	40	320	40	<20	320	40	40	<20	40	40	320
			22	1280	160	160	<20	160	160	1280	<20	160	1280	160	<20	1280	80	160
	SECOND	DA	8	160	640	80	<20	640	40	160	<20	160	160	640	<20	160	640	40
2	FIRST	MUMPS	14	320	80	160	<20	40	160	160	<20	160	160	80	<20	320	80	160
			22	640	80	160	<20	80	160	640	<20	160	640	160	<20	640	80	160
	SECOND	DA	8	80	640	40	<20	640	40	80	<20	80	80	640	<20	80	640	40
	THIRD	MUMPS	11	2S60	40	160	320	40	80	2S60	<20	80	2S60	40	<20	2S60	40	80
3	FIRST	MUMPS	11	160	40	1280	<20	40	1280	160	<20	640	160	40	<20	80	80	640
			22	2S60	40	1280	<20	40	640	1280	<20	640	2S60	80	320	1280	40	640
	THIRD	MUMPS	11	2S60	80	640	160	160	1280	2S60	<20	1280	2S60	40	320	2S60	80	640
4	FIRST	MUMPS	14	80	40	320	<20	40	320	80	<20	160	160	40	<20	80	40	320
			22	1280	20	320	<20	20	160	1280	<20	160	1280	20	<20	1280	40	160

Table 2. Results of antibody absorption of sera from monkeys #1-4.

MONKEY NO.	VIRUS INOC.	DAYS AFTER INOC.	CONTROL			ABSORB c̄ MUMPS			ABSORB c̄ DA			ABSORB c̄ PARA-3			ABSORB c̄ NDV		
			M	DA	P-3	M	DA	P-3	M	DA	P-3	M	DA	P-3	M	DA	P-3
S	MUMPS	0	<20	80	160	<20	80	160	<20	<20	160	<20	160	<20	<20	80	160
		7	40	80	160	<20	80	80	40	<20	80	40	80	<20	40	160	160
		21	40	80	160	<20	80	80	40	<20	160	40	160	<20	40	80	80
6	MUMPS	2	40	320	40	<20	320	20	40	<20	80	40	320	<20	40	320	80
		7	80	160	40	<20	160	40	80	<20	80	40	160	<20	80	320	20
		21	160	320	80	<20	320	40	160	<20	80	160	640	<20	160	640	40
7	MUMPS	0	20	40	40	<20	40	40	20	<20	40	20	40	<20	20	80	40
		7	20	40	40	<20	40	40	40	<20	80	40	40	<20	40	40	20
		21	160	80	80	<20	40	20	160	<20	80	160	160	<20	160	80	320
CHIMPS 50	DA	3	20	160	320	<20	160	320	20	<20	320	20	320	320	20	160	320
		60	40	640	320	<20	640	320	40	<20	320	40	640	80	40	640	320
		120	20	1280	320	<20	640	320	20	<20	320	40	1280	80	40	1280	320

Table 3. Results of antibody absorption of sera from monkeys #5-7.
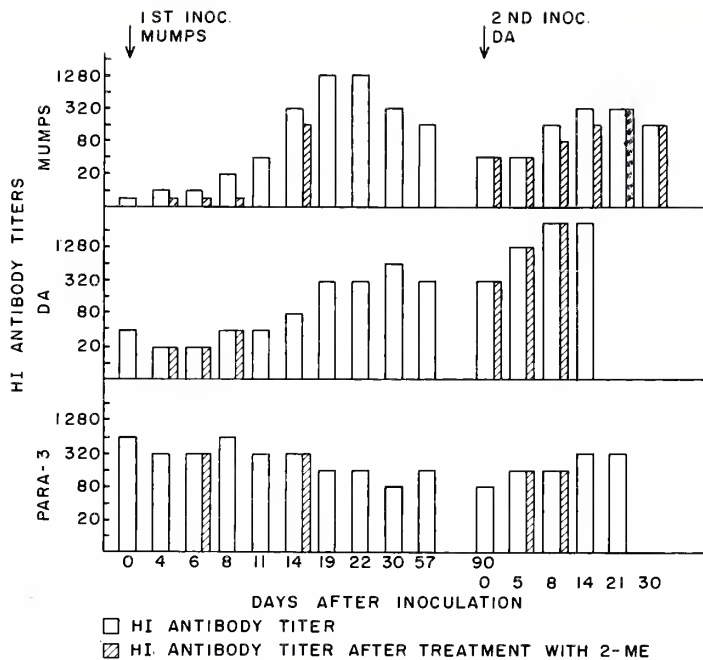
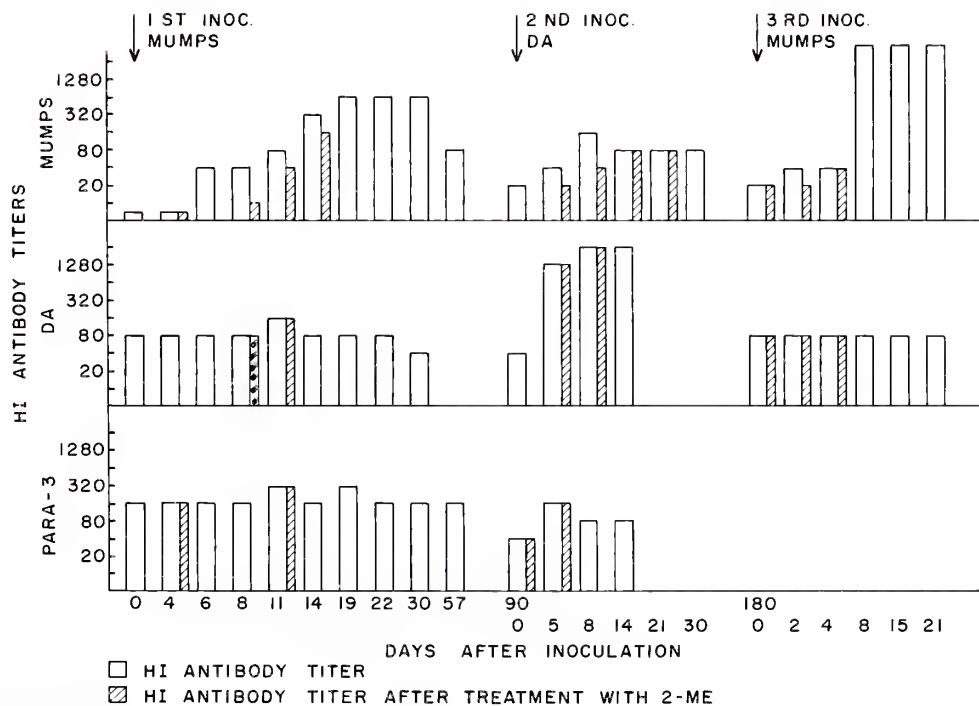Figure 1.   Monkey #1.   HI antibody titers following virus inoculations.

Figure 2.   Monkey #2.   HI antibody titers following virus inoculations.
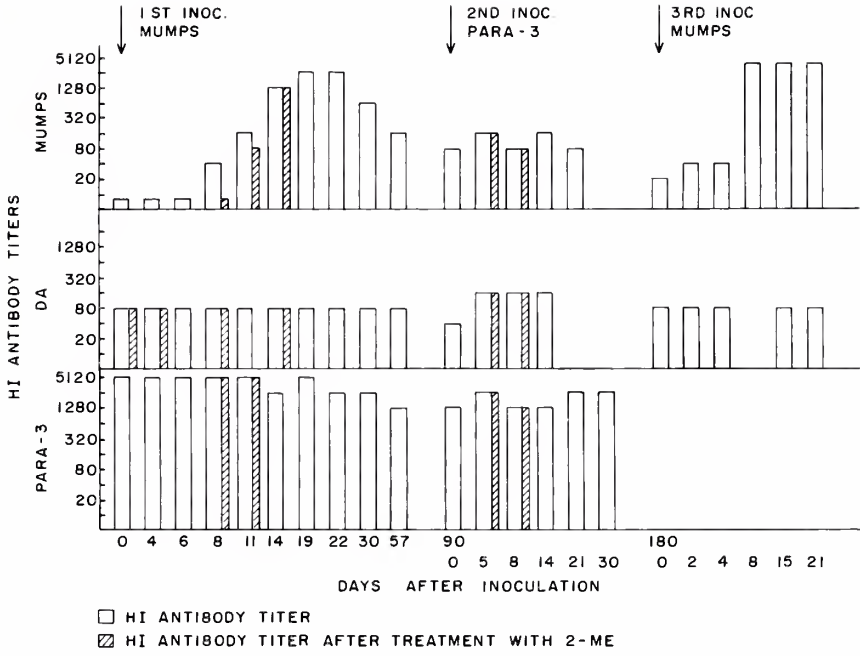
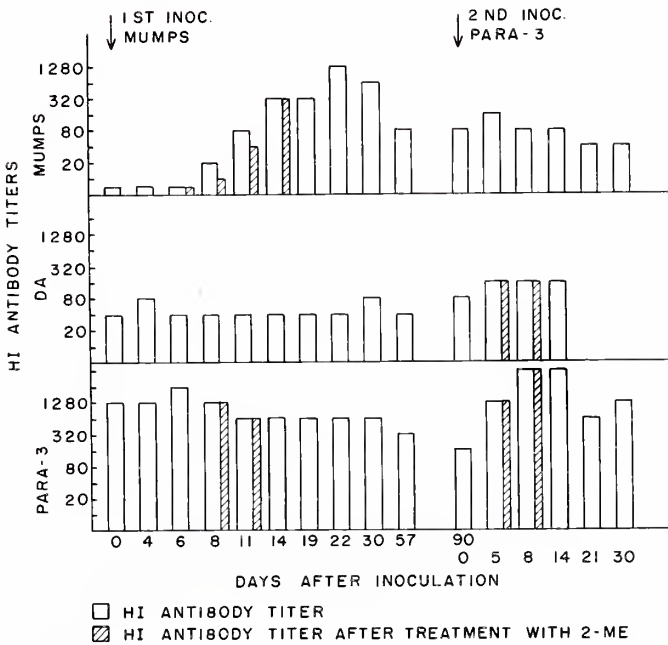Figure 3. Monkey #3. HI antibody titers following virus inoculations.

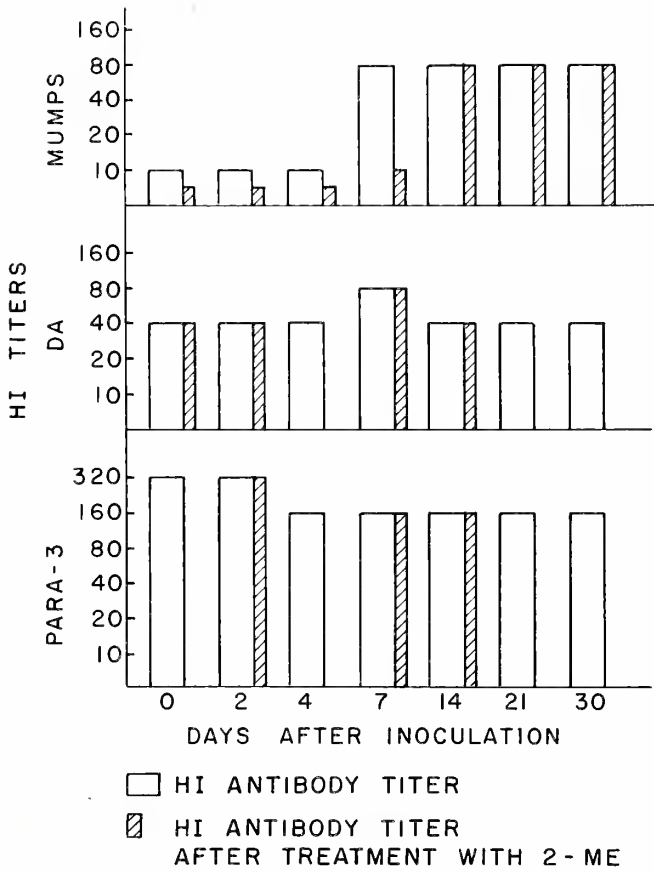Figure #4. Monkey #4. HI antibody titers following virus inoculations.

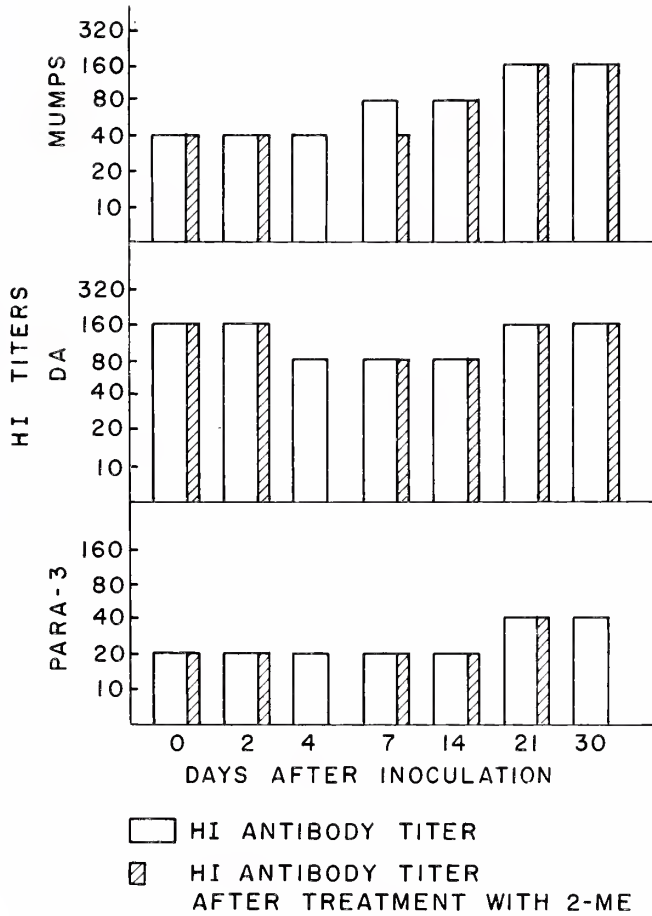Figure 5.   Monkey #5.   HI antibody titers following mumps inoculation.

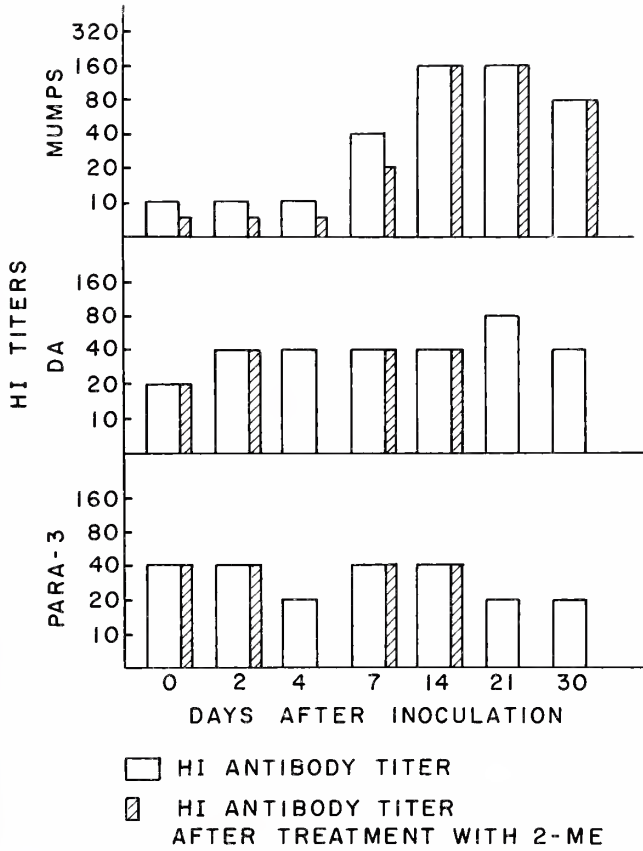Figure 6.   Monkey #6.   HI antibody titers following mumps
inoculation.

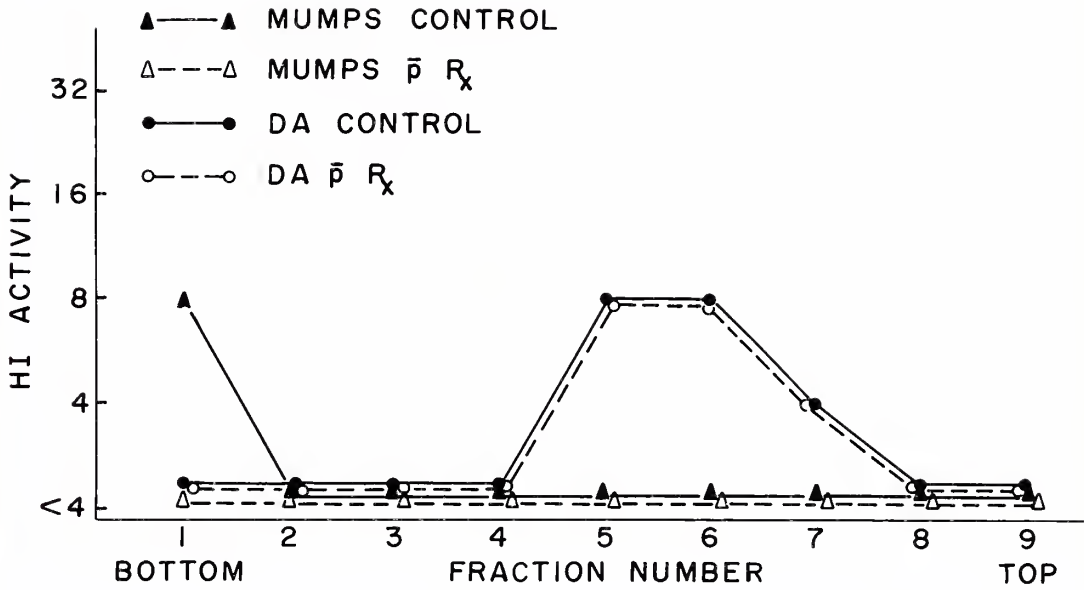Figure 7. Monkey #7. HI antibody titers following mumps inoculation.

Figure 8. Monkey #1 eight days after first mumps inoculation. Fractionation of serum in symmetric sucrose gradient.
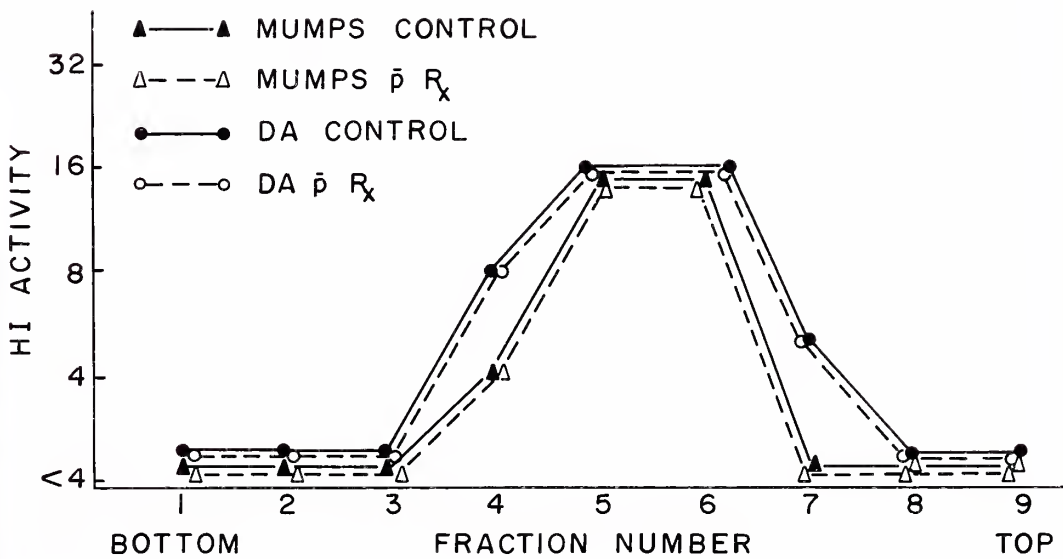

Figure 9. Monkey #1 ninety days after first mumps inoculation. Fractionation of serum in symmetric sucrose gradient.
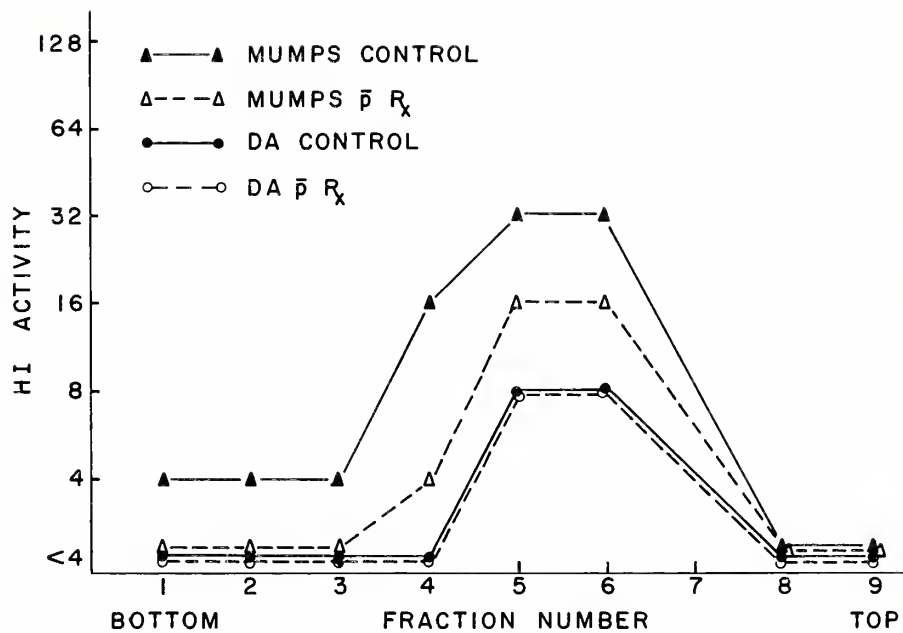
Figure 10.   Monkey #1 eight days after DA inoculation.
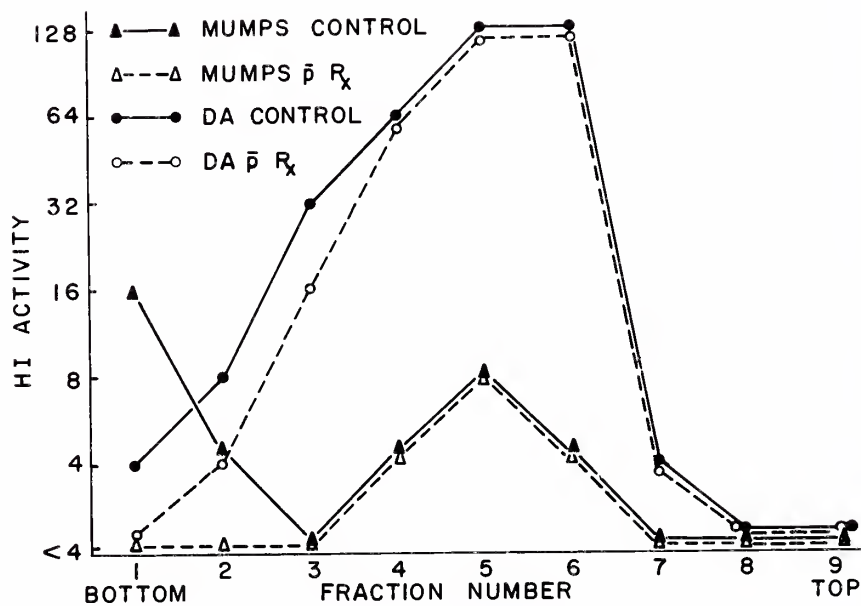Fractionation of serum in symmetric sucrose gradient.

Figure 11.   Monkey #1 eight days after DA inoculation.
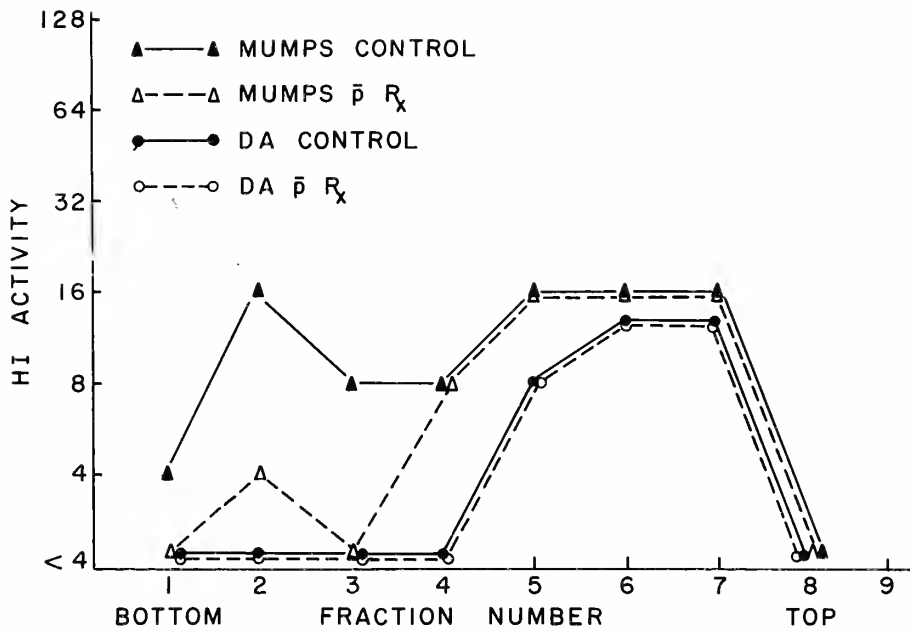Fractionation of serum in assymmetric sucrose gradient.

Figure 12.   Monkey #2 fourteen days after first mumps inoculation.
Fractionation of serum in symmetric sucrose gradient.
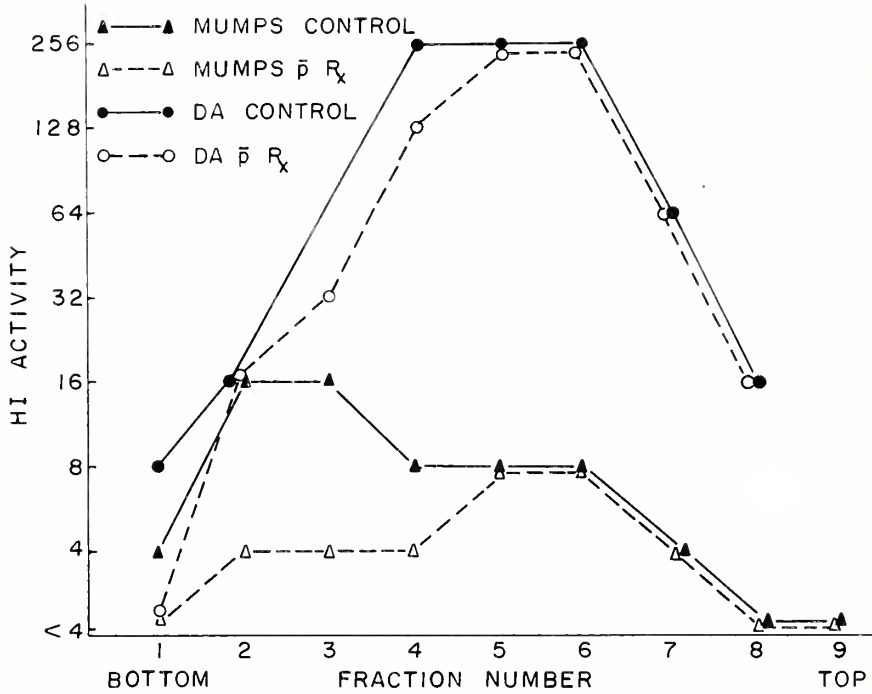
Figure 13. Monkey #2 eight days after DA inoculation. Fractionation of serum in symmetric sucrose gradient.
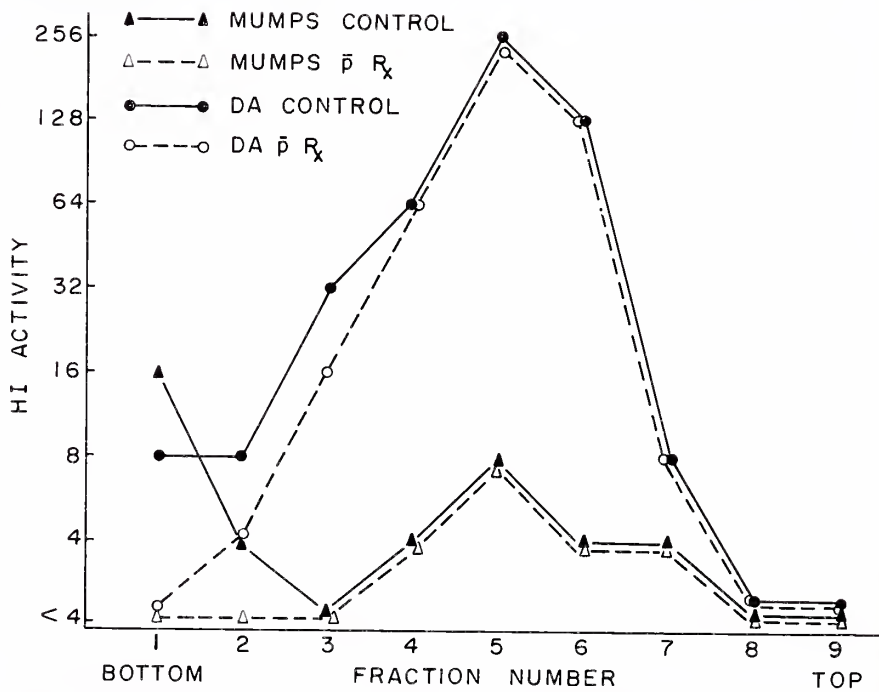

Figure 14. Monkey #2 elght days after DA inoculation. Fractionation of serum in asymmetric sucrose gradient.
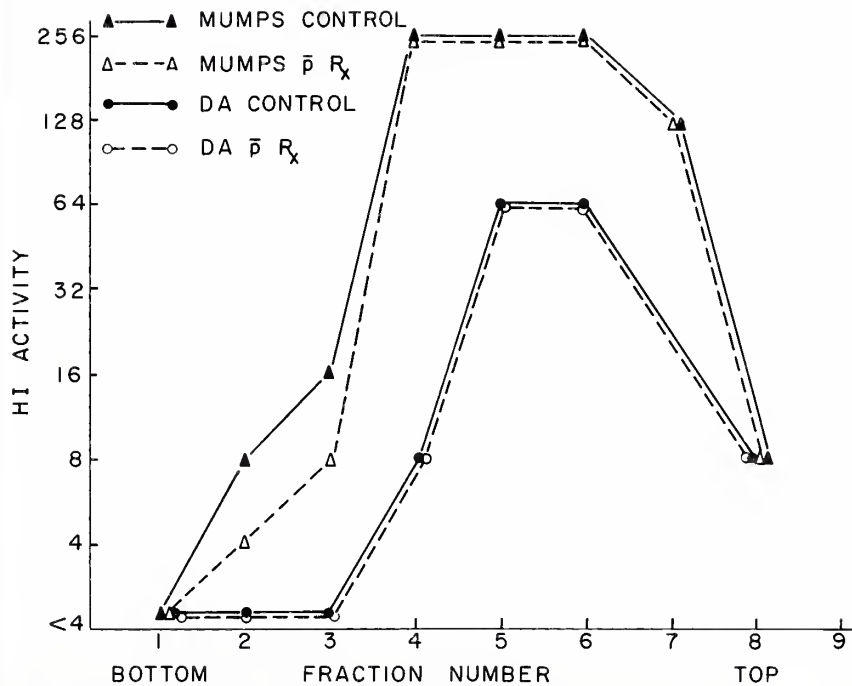
Figure 15. Monkey #2 eight days after second mumps inoculation. Fractionation of serum in symmetric sucrose gradient.
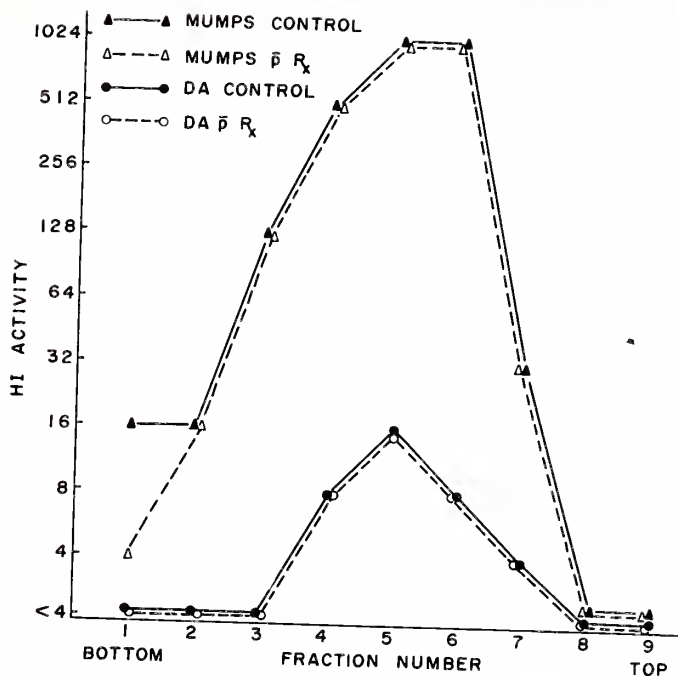

Figure 16. Monkey #2 eight days after second mumps inoculation. Fractionation of serum in asymmetric sucrose gradient.

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