

## IMMUNITY IN MUMPS

### I. EXPERIMENTS WITH MONKEYS (*MACACUS MULATTA*). THE DEVELOPMENT OF COMPLEMENT-FIXING ANTIBODY FOLLOWING INFECTION AND EXPERIMENTS ON IMMUNIZATION BY MEANS OF INACTIVATED VIRUS AND CONVALESCENT HUMAN SERUM\*

By JOHN F. ENDERS, Ph.D., LEWIS W. KANE, M.D., SIDNEY COHEN, M.D.,  
AND JEANETTE H. LEVENS

(From the Department of Bacteriology and Immunology, Harvard Medical School and School of Public Health, Boston)

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Mumps as a disease of military personnel may be exceedingly troublesome, causing loss of much time from duty and interfering seriously with the movement of troops (1, 2). This fact induced us to undertake the experiments which are described in this and the following papers with the hope that ways may be found by which epidemics can be controlled.<sup>1</sup>

The fundamental researches of Johnson and Goodpasture (4-7) demonstrated the causative agent of mumps to be a filterable virus and revealed certain important facts concerning passive and active immunization of the monkey against experimental inoculation. Their investigations, however, left unsolved a number of practical problems. Among the more important are: (a) the development of specific methods of diagnosis, particularly in doubtful cases in which parotitis is absent or is not a prominent feature; (b) the determination of a convenient method for the revelation of susceptibility or resistance among human beings—especially among those who have never experienced an apparent illness but of whom some may have had the disease in an inapparent or “silent” form; (c) the discovery of a means of effectively conferring active immunity upon susceptible individuals; (d) the development of methods for the assay of the protective properties of convalescent sera for the value of which some clinical evidence has been presented (8, 9).

In the present paper experiments on monkeys (*M. mulatta*) are described<sup>2</sup> which gave results that seem significant for the solution of certain of these

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<sup>1</sup>Wesselhoeft and Walcott (3) have recently emphasized the importance of mumps as a military disease and discussed its control.

<sup>2</sup>A preliminary report of certain of these observations has been published (10, 11).

questions and which represent the basis for the investigations in human beings to be subsequently reported. The aims of this experimental work were: (1) to establish an infection with mumps virus in *M. mulatta* and to maintain the virus by serial passage, (2) to determine whether evidence of humoral immunity could be obtained following the recovery of this animal from mumps, (3) to ascertain whether active immunity could be induced by inoculation of material containing the virus which has been inactivated by suitable procedure, (4) to determine whether human convalescent serum possessed neutralizing properties for the virus of mumps.

### *Materials and Methods*

*Monkeys.*—*M. mulatta* has been exclusively employed. Animals of different size and age have all been successfully inoculated. Furthermore, no difference in susceptibility associated with sex was observed.

*Virus.*—Initial infection of the monkey was accomplished by the method of Johnson and Goodpasture (4). Saliva was collected from patients with the classical signs of mumps during only the 1st or 2nd day of the disease. Both Johnson and Goodpasture (4) and Findlay and Clarke (12), who confirmed the work of the American authors, were unable to produce infection with specimens taken after the 2nd day. The patient was induced to expectorate into a container at intervals during a period of about 2 hours. The saliva was either immediately brought to the laboratory or kept overnight in the ice box before transportation when it was then mixed with an equal quantity of infusion broth. The mixture was either inoculated at once or stored for varying periods in a CO<sub>2</sub> ice cabinet. About 2 ml. of the mixture was injected into the parotid gland of a monkey *via* Stensen's duct, employing for this purpose a 24 gauge hypodermic needle from which the point had been removed.

The gland was usually excised on the 4th or 5th day following inoculation. Since it was desired to preserve the monkeys for subsequent study, parotidectomy was carried out under ether or nembutal anesthesia. The animals usually made an uneventful recovery with merely paralysis of certain of the muscles of the cheek and eyelid as permanent residua. Both glands were removed during the same operation with no incapacitating sequelae.

The gland was at once weighed, minced by means of scissors, and ground in a mortar with alundum. Sufficient saline (0.85 per cent NaCl) was added to give a suspension containing 20 per cent of tissue. The material may be stored in glass-sealed pyrex ampoules in a carbon dioxide ice cabinet where its capacity to infect *M. mulatta* is preserved for 10 months.

For passage, the 20 per cent suspension was usually diluted with one to five parts of infusion broth and the mixture inoculated as described above. One ml. of a 0.6 per cent suspension of gland has caused infection in the monkey. The minimal infecting dose has not been determined.

*Materials of the Complement Fixation Test.*—All dilutions are recorded as the final dilution of the reagent before the addition of sensitized cells unless otherwise indicated. This notation appears to be desirable for reasons which one of us has previously emphasized (14). The materials and technique employed in the complement fixation test are essentially the same as previously described (10). Certain modifications have been introduced with the purpose of conserving antigen.

*Preparation of Antigen.*—One volume of a 20 per cent suspension of parotid is diluted with 5 volumes of saline and rotated for 30 minutes at 3500 R.P.M. in an angle centrifuge. The lipoidal material which floats on the surface and the sediment are discarded. The supernatant fluid was used as antigen in most of the earlier experiments reported in this and the fol-

lowing two papers. It is now, however, our practice to determine the capacity of a given glandular suspension to fix complement in the presence of either monkey or human serum known to contain antibody, since it has been found that various lots may vary considerably in their content of antigen. This is accomplished by determining the highest dilution in saline of the original suspension of gland which gives complete fixation with 3 strongly positive convalescent sera in dilutions of 1:24. As controls for non-specific fixation 3 sera known not to contain antibody are included to which are added the same dilutions of suspension. The positive sera must give "2+ fixation" in a dilution of at least 1:384 with an antigen diluted 1:113 or one which has been previously standardized by this method. For use in the complement fixation test, twice the concentration of antigen giving complete fixation with at least 2 of the 3 positive sera in the titration is employed.

To control further the specificity of the reaction an emulsion of the parotid gland from a normal monkey prepared in the same manner as the infected parotid is employed. This is used in a dilution equivalent to that of the infected parotid.

*Sera.*—Blood obtained from the femoral vein of the monkey is allowed to clot; the serum is collected and heated at 62°C. for 20 minutes to inactivate complement and remove non-specific complement-fixing properties. Following the method of Casals and Palacios (13) when human sera are used, they are heated at 60°C. for 20 minutes. For preservation, sera are usually stored at 4°C. in tubes closed with rubber or cork stoppers. If monkey sera are anticomplementary, it has been found that this property may be completely removed in most instances by heating the serum at 62°C. for 20 minutes, preparing the requisite dilutions and, after allowing them to stand overnight at 4°C., heating them again at 62°C. for 20 minutes. This procedure has been found not to affect the antibody content of the serum. Human sera are double heated at 60°C. It is usually possible to remove the anticomplementary effect by double heating without dilution, allowing a 2 hour interval at 4°C., but this procedure is occasionally not as effective.

*Hemolytic System.*—To one volume of a 2 per cent suspension of thoroughly washed sheep's cells, one volume of diluted anti-sheep cell rabbit serum containing 2 units of amboceptor is added 15 minutes before the sensitized cells are used in the test.

*Complement.*—Sera from at least 6 guinea pigs are collected and pooled. This material is preserved in the CO<sub>2</sub> cabinet until exhausted. No diminution in complement titer has been observed under these conditions during a period of at least 2 months. The complement is always titrated on the day of the test.

*The Complement Fixation Test.*—Appropriate twofold dilutions in saline of the inactivated serum to be tested are prepared. Usually these dilutions range from 1:6 to 1:384. To 0.125 ml. of each dilution of the serum to be tested, 2 units of complement are added in a volume of 0.15 ml. and then 0.1 ml. of the antigen. The mixtures are placed overnight in the ice box at 4°C. and 0.25 ml. of sensitized sheep's cells added to each the following morning. Results are recorded after one half hour at 37°C. in the water bath. The endpoint is taken as the last dilution giving definite fixation of complement denoted as 1+. Complete fixation is denoted as 4+.

The following controls are included in each test:

1. Unknown serum alone (lowest dilution used in test)
2. " " and 2 units of complement
3. " " " 1,3 " " "
4. 2 units of complement
5. 1 unit " "
6. Unknown serum, 2 units of complement, and normal parotid gland suspension
7. Normal parotid gland suspension and 2 units of complement
8. " " " " " 1,3 " " "
9. " " " " " " " "

10. Infected parotid gland suspension and 2 units of complement
11. " " " " " 1.3 " " "
12. " " " " " " " "
13. Salt solution
14. A number of dilutions of a known positive serum sufficient to cover the endpoint range. To these are added infected parotid gland suspension and 2 units of complement. The usual controls for the anticomplementary effect of the serum and the normal parotid gland suspension are included.
15. A known negative serum diluted 1:6, infected parotid gland suspension, and 2 units of complement.

After the addition of saline to bring the volumes to equivalence with those of the test, the control tubes are maintained under the same conditions and each receive 0.25 ml. of sensitized sheep's cells.

#### EXPERIMENTAL

##### *Mumps in M. mulatta*

*Infection of the Monkey with Saliva from Cases of Mumps.*—The disease which we have produced in *M. mulatta* resembles in all essential respects that described by Johnson and Goodpasture (4) and by Findlay and Clarke (12). Evidence of infection has been obtained following the inoculation of 5 batches of saliva collected in the city of Boston.

The first 4 specimens were derived from 4 different individuals, the last consisted of a pooled saliva supplied by 4 patients. The criteria of infection consisted in:

(1) Definite swelling of the parotid gland after 5 to 8 days; (2) transfer of the disease to another animal by inoculation of the parotid tissue; (3) demonstration of specific complement-fixing antigen in the infected parotid; (4) demonstration of the development or presence of complement-fixing antibody in the serum during convalescence.

Infection was demonstrated in the case of the first specimen by criterion 4, in that of the second by all 4 criteria. This latter strain of virus designated as the stock virus has been employed almost exclusively throughout the course of our investigation. In the third specimen of saliva the virus was revealed by criteria 1 and 4, in the fourth by Nos. 3 and 4, and in the fifth by No. 4. In addition to this evidence for the nature of the virus with which we worked is the fact that both experimental and accidental mumps in the human being has resulted from inoculation of material from the 11th monkey passage of the second strain (15, 16).

Complement fixation tests employing 1 strain of virus as antigen and convalescent sera from monkeys infected with the first 4 specimens of saliva mentioned above have given no indication that any of these viruses possessed different antigenic characteristics capable of being revealed by this reaction. It is true that the specimens were collected in one geographical area but, as will be shown in subsequent communications (15, 17), our stock virus caused the fixation of complement in the presence of high dilutions of sera obtained from epidemics of mumps which occurred in Wisconsin and in Philadelphia. These findings, together with the fact that second attacks of the disease are rare, suggest that the virus may consist of a single antigenic species.

*Signs of Infection in the Monkey.*—The gross signs of parotitis which resembled those described by Johnson and Goodpasture (4), consisted of enlargement of the gland as determined by palpation and edema of the surrounding tissues. These manifestations have been observed by us to occur in a group of 5 monkeys from the 5th to the 8th day after inoculation. Following the establishment of serologic criteria of infection, it has been our usual practice to remove the glands on the 5th or 6th day after inoculation. We have, therefore, relatively few animals in which these symptoms have been allowed opportunity for greatest development. Our observations, however, are sufficient to indicate that these signs in different animals may vary in their intensity and occasionally may be so ill defined as to leave the observer in doubt as to whether infection is present. Minimal edema and parotid hypertrophy have been in our experience characteristic of the infection following the inoculation of saliva derived from cases of mumps. In only 3 animals which received saliva, however, has the period of observation been adequate.

In accordance with the statement of Findlay and Clarke (12), we have not remarked in monkeys, inoculated into 1 gland only, obvious signs of involvement of the uninoculated contralateral gland. In 1 animal, however, inoculated into the left parotid only, complement fixing antigen was demonstrated in considerable quantity in both glands. This single observation can merely suggest that spontaneous infection of the uninoculated gland may occasionally ensue.

*Weight and Size of the Infected Gland.*—The weights of infected parotid glands have varied considerably. From 18 monkeys, 29 parotids have been weighed on an average of 5.2 days after inoculation. All these glands were shown to contain virus either by subsequent inoculation into a normal animal or by the demonstration of the antigen.

The smallest gland weighed 2.1 gm. and the largest 9 gm. The mean weight was 5.2 gm. In 10 animals of the group, both glands were inoculated. In these cases the glands from the same animal were found to be of approximately the same weight. Many were excised on the 4th or 5th day following the inoculation and accordingly may not have reached the state of maximal enlargement. However, an infected gland removed on the 7th day weighed only 2.6 gm.; 3 on the 6th day weighed 2.3, 2.8, and 3.2 gm. respectively. In contrast the largest gland encountered, 9 gm., was obtained on the 5th day. That the weight of the infected gland may not be significantly increased at a time when much virus is presumably present was shown by an experiment in which only 1 gland in a pair of monkeys was inoculated but both glands removed from each on the 6th postinoculative day. The inoculated gland from 1 weighed 2.3 gm. and the uninoculated gland 2.6 gm.; the corresponding glands from the other weighed 2.8 and 2.1 gm. respectively. In the uninoculated glands no antigen could be demonstrated, whereas in the inoculated glands titers of 1:1200 and 1:600 were observed. We must conclude, then, that the *weight* may not increase markedly following infection although in most instances the *size* of the organ, as judged by palpation, appears to do so.

Again these observations are not in complete agreement with those of Johnson and Goodpasture (4) who recorded a regular increase in weight of infected glands. In our experience there has been a more definite correlation between the *size* of the animal and the weight of the gland than between the infected state and weight. For the production of the maximal quantity of virus, therefore, one should select large animals. This fact from a practical standpoint is of importance. Since the parotid gland of the monkey is so far the only available source of the mumps virus, and since considerable amounts of material for antigen and possibly vaccine may be required (16), it is essential that the optimal yield be obtained from each animal.

*The Quantity of Antigen in the Parotid Gland at Various Periods after Inoculation.*—The summary of a number of titrations of parotid suspensions given in

TABLE I  
*Titers of Complement-Fixing Antigen in Parotid Glands of M. mulatta*

Days after infection				
4	5	6	7	8
290	600}	113}		290
>225	150}	150}		
150}	150}	>225*	150*	
150}	600}	1,200		
150}	290}	600		
150}	290}	150}		
	290	150}		
	600}	600		
	600}	150		
	290}	1,200		
	290}	600		
	290}	150*		
	75*	106}		
	290}	150}		
	290}	600		
	150}			
	290}			
	290}			
	150}			
	150			
	2,400}			
	1,200}			
	>800			
	600}			
	600}			
	290}			
	290}			
	600}			
	600}			
	213}			
	290}			
	290}			
	150}			

The titer is expressed as the reciprocal of the highest dilution of parotid suspension giving complete fixation with excess antibody.

The bracketed figures are the titers obtained from the left and right glands of the same animal.

\* Glands removed from the same animal.

Table I shows that the complement-fixing antigen may be present in considerable amount within 4 days after inoculation of the gland. The maximal concentration is apparently attained in most instances 5 days after inoculation.

At this time, it should be emphasized, the fully developed signs of mumps were not yet present in most animals. Although we cannot state whether or not viral infectivity parallels concentration of complement-fixing antigen, a quantitative relationship of this sort would seem on *a priori* grounds to be probable. If it does obtain, the maximal quantity of virus would have developed in the gland at a time previous to the occurrence of the most intense reaction on the part of the host's tissues. Phenomena of this sort have been observed with other viruses. For example, the infective and complement-fixing titers of influenza A virus reach maxima in the lungs of mice 2 to 3 days after inoculation whereas the pneumonic consolidation is not marked before the 4th or 5th day (18, 19).

The results recorded in Table I also are of interest since they make it clear that very considerable quantities of the antigen may be produced. Thus complete fixation of complement has been obtained with one suspension diluted 1:2400 and glands from a number of other animals gave yields only slightly less. The average titer of the glands excised on the 5th and 6th days is about 1:400.

We have alluded to the presence of the complement-fixing antigen in the gland with the implication that the detection of this factor constitutes evidence of infection. In view of the facts just presented taken together with observations which will be described, there would seem to be no ground for questioning this assumption since (*a*) the antigen has been found only in animals after inoculation with the virus, (*b*) has never been demonstrated in other tissues of infected monkeys such as spleen, pancreas, brain, and testicle or (*c*) in the parotid gland of normal animals, and (*d*) reacts specifically with an antibody which is not present in normal animals, but which appears regularly during convalescence.<sup>3</sup>

*The White Blood Count.*—Johnson and Goodpasture (4) noted a decrease in the leucocytes with a relative and absolute increase in the number of round cells. These changes occurred just before the development of local signs. We have performed daily white counts and a few differential counts on a number of animals.

In a group of 5 monkeys definitely shown to have undergone an infection some fall in the total white cell count was observed in every case. This usually occurred just before and during the time of obvious mumps, that is, from the 6th to the 8th day after inoculation although in 3 animals some decrease was noted as early as the 3rd or 4th day. In 2 instances the decrease was about 50 per cent of what might be regarded as the normal count; in the other 3 it was only of the order of 20 to 30 per cent. One animal which was reinoculated with active virus following recovery, and in which white blood counts were taken, also showed a slight but definite decrease on the 5th day. Another monkey inoculated with egg material gave no definite evidence of infection yet exhibited a decrease in the total white count of 35 per cent on the 3rd and 4th days.

Because of these findings and additional observations made during a study of measles in the monkey on the marked variation which may occur in the total and differential counts of the

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<sup>3</sup> See page 100.

normal animal, we conclude that although infection with the virus often may induce a fall in the number of circulating white blood cells, the determination of the count is of little value as a criterion of infection. Accordingly in our more recent experiments we have not sought it as an aid in diagnosis, depending rather on the demonstration of antigen in the gland or antibody in the serum.

*Rise in Body Temperature.*—Equally unsatisfactory has been our experience with a rise in body temperature as an index of infection. In only 1 animal of 14 in which daily temperature readings were recorded and in which either the antigen or development of antibody was demonstrated did the temperature reach 104°F. Moreover, excepting in that single animal, the difference in readings for each individual was not greater than about 1.5°F. Johnson and Goodpasture, however, frequently noted temperatures of 104°F. during the course of the infection. The discrepancy may possibly be due to the existence of strains of virus differing in pyrogenic capacity; but in any event we are inclined to regard a rise in body temperature, like leucopenia, as irregularly associated with the disease.

*Histology of the Inoculated Gland.*—Johnson and Goodpasture (4) and Bloch (20) have described the histologic changes induced in the parotid gland as a result of experimental infection with the virus. We have examined the glands from 7 monkeys removed at periods varying from 5 to 9 days following inoculation. Included in this series was 1 animal which received a filtered suspension of infected parotid shown to be sterile by suitable methods of cultivation. In every instance except in the gland removed on the 9th day when obvious signs of mumps had almost completely subsided, all the appearances considered by the authors just mentioned as being characteristically caused by the activity of the virus were observed. Glands from 4 normal monkeys and from 1 animal inoculated with the virus of poliomyelitis did not exhibit these pathological changes.

#### *The Development of Complement-Fixing Antibody Following Inoculation with the Virus of Mumps*

In a preliminary report (10) evidence has been presented which shows that antibody capable of fixing complement in the presence of a suspension of the infected parotid gland appears in the serum of monkeys recently convalescent from experimental mumps. Since the publication of this communication, a large number of animals have been studied. The results of tests on the sera of 11 monkeys bled before inoculation with the virus and at various intervals thereafter are presented as examples in Table II. From this material and many other determinations not recorded in detail, several facts emerge:—

*The Absence of Antibody in the Sera of Normal Monkeys and the Occurrence of Inapparent Contact Infections.*—The complement-fixing antibody is not usually present in the sera of monkeys before inoculation with the virus. The sera obtained from 47 presumably normal monkeys were tested. Even in low dilutions (1:6 or 1:12) no antibody was demonstrated in 42 animals. With the sera of the remaining 5 animals, fixation occurred.

But it was not until many monkeys had been studied that antibody was encountered in an animal which was supposedly normal. Since the antibody, however, appeared to be specific and since it was known that the animals most recently studied had been housed for considerable periods of time in the same quarters with monkeys infected with the virus, it seemed possible that certain

of these contacts might have undergone inapparent infections. An experiment was undertaken in which a normal monkey of a recent shipment was kept in the same cage with an animal which had been inoculated with virus. Complement-fixing antibody could not be demonstrated in the serum of the normal

TABLE II  
*The Development of Complement-Fixing Antibody in the Serum of M. mulatta Following Infection with the Virus of Mumps*

Monkey	Test done before inoculation or during acute stage		Test done during early convalescence		Test done after recovery		Remarks
	Time after inoculation	Titer*	Time after inoculation	Titer‡	Time after inoculation	Titer‡	
	<i>days</i>		<i>days</i>		<i>months</i>		
1	0	0	22	2170‡	2	275	
43	0	0	13	1024	7	96	
19-37	7	0	18	1024	3	185	
5	0	0	13	1536	3	185	
21	0	0	13	3072	3	192	
22	0	0	13	384	3	<6	A 1:24 dilution of serum gave a tr reaction with the 3 mo. specimen.
19-35A	0	0	19	>1024	1	1024	A 3+ reaction was obtained with early convalescent serum in dilution recorded.
19-38	0	0	—	nd	1½	185	
6	—	nd§	18	1095	10	24	
17	—	nd	—	nd	4	12	
19-34	—	nd	—	nd	21	17	This animal was inoculated with saliva. Titer 3 mos. after inoculation was 180.

\* In the majority of cases initial dilutions of 1:2 were tested. In the other instances initial dilutions of 1:4 were the lowest tested.

‡ Reciprocal of final dilution of serum giving 1+ fixation. In certain instances the end-point was calculated by determining the geometrical mean between the dilution giving greater fixation than 1+ and the next lower dilution giving less than 1+.

§ nd, not done.

monkey before the experiment was begun. Contact was maintained during the 5 days following the inoculation of the other monkey when the normal monkey was placed in a cage by itself and was observed for 50 days. During this time specimens of its serum were tested. No indication of disease was noted but antibody was found in the serum 34 days after initial exposure. It was not present on the 3rd, 5th, 6th, 9th, and 19th days after exposure.

Following challenge inoculation with virus, antigen was not revealed in the glands. This result indicates that in the monkey contact infection can occur which is inapparent in form but leads to the emergence of antibody and the establishment of resistance. After this experiment was completed, 2 monkeys kept in the same room with recently infected animals developed antibody. They too proved refractory to inoculation of virus.

Johnson and Goodpasture (4) did not observe any signs of disease in their normal animals which were in contact with those which had been inoculated, and so concluded that infection did not take place in this manner. From our experience it would seem that under such conditions infection does occur. The necessity, therefore, of maintaining infected animals in quarters separate from those occupied by normal monkeys is obvious.

*Time of the Appearance of Antibody and Its Persistence Following Recovery.*—Antibody may appear in the serum within a short time after the virus is injected. Thus in 1 animal a titer of 1:96 was demonstrated on the 8th and in another on the 9th day. In other cases such as M19-37 alluded to in Table II, no antibody was demonstrable in the late acute stage. By the 13th to 15th day, however, the antibody is usually present in high concentration and at about this time the maximum is probably attained as is indicated by the results summarized in Table II and the records of successive determinations of antibody in the same animal such as that presented below in the protocol for monkey 20-47. The protocol also reveals that a rapid and extensive increase in concentration occurs once the antibody has made its appearance. Similar abrupt rises in antibody levels have been noted in certain human beings during the convalescence and will be reported in the second paper of this series (15).

*Protocol 1.*—Monkey 20-47. Mar. 30, 1944. Inoculated into both ducts of Stensen with 1 ml. of 1:40 dilution of suspension of infected gland of the 11th monkey passage. Apr. 4, 1944. Both glands removed under ether anesthesia. The right gland completely fixed complement in a dilution of 1:300 and the left in a dilution of 1:150. At various times, samples of serum were taken and titrated for antibody.

Date blood drawn	Reciprocal of highest dilution of serum giving fixation of complement
3/23/44	No fixation (serum diluted 6)
3/31/44	" " ( " " " )
4/ 4/44	" " ( " " " )
4/ 5/44	" " ( " " " )
4/ 6/44	" " ( " " " )
4/ 7/44	96
4/10/44	>96 <128
4/11/44	384
4/13/44	>384 <1536
4/15/44	>384 <1536
4/17/44	>384 <1536
4/19/44	>384 <1536

Date blood drawn	Reciprocal of highest dilution of serum giving fixation of complement
4/24/44	1536
4/28/44	1536
5/ 2/44	384
5/ 5/44	384
5/12/44	384
5/20/44	96
5/25/44	96
7/31/44	>48 <96
8/29/44	>48 <96

A high concentration of antibody may be retained for about 1 month; but by the 2nd or 3rd month after inoculation the titers usually declined from the early convalescent level by at least a factor of 5.

From the results obtained with the sera of monkeys 6 and 19-34 (Table II) and M 20-47 (protocol), it is evident that antibody may persist for many months. The titers of antibody recorded at 10 and 21 months are of approximately the same magnitude as those encountered in the sera of normal human beings who give positive complement fixation tests (17). Furthermore, they are comparable to the titers of the sera of most persons convalescent from mumps which have been drawn at analogous periods following the disease (15).

*The Regularity of Antibody Formation Following Infection.*—Following the intraparotid inoculation of virus, antibody has appeared *de novo* in the serum of 19 normal monkeys which have been studied. In no instance have we failed to demonstrate its formation when the test has been carried out at the appropriate time. This uniformity of response shows that the existence of antibody in the monkey is a reliable index of previous infection. From the results of similar studies on mumps in human beings (15), the same regularity of antibody formation has been demonstrated.

#### *Properties of the Complement-Fixing Antigen*

*Thermostability.*—Experiments have shown that the antigen in suspensions of infected glands responsible for the fixation of complement in the presence of the specific antibody is moderately resistant to heat. The thermostability of the antigen at two temperatures in varying hydrogen ion concentrations was investigated.

Suspensions of infected gland (3.3 per cent) were prepared in a series of isotonic phosphate buffers at pH 5.2, 5.9, 6.4, 6.7, and 7.5. The preparations were maintained at 37°C. for 2 hours when flocculation of protein was observed at pH 5.2. After adjustment of the reaction to pH 7, a portion of each preparation including the precipitate and supernatant fluid of that at pH 5.2 was tested for capacity to fix complement. Other portions were heated for an additional period of 20 minutes at 65°C. Under these conditions a varying degree of flocculation was noted in all, being most abundant at the more acid reactions. The precipitates were resuspended in buffer at pH 7 and the supernatant fluids adjusted to the same hydrogen ion concentration. They were then employed as antigens in the complement fixation test. The results showed that in the range pH 6.4-7.5 the antigen, all of which remained in the supernatant fluid, was unaffected at 37°C. for 2 hours followed by further heating at 65°C. for 20

minutes. At the most acid reaction activity was reduced at 37°C. but was not entirely eliminated at 65°C. This residual activity was associated with the precipitated protein.

*Maintenance of Activity during Storage.*—After standing for 1 week at room temperature (18–20°C.), the original titer of a 20 per cent suspension of the gland was reduced about one-half. The titer of a 20 per cent suspension remained unchanged for 12 months in the CO<sub>2</sub> ice cabinet. The titer of a 6.6 per cent suspension of the gland also remained constant 16 days at 4°C. and was only slightly reduced after 45 days. It is therefore unnecessary to prepare the antigen for each test from material which has been stored in the CO<sub>2</sub> cabinet.

*The Effect of Formalin.*—In view of the possible use of formalized virus as a vaccine, the effect of various concentrations of commercial formalin on the activity of the antigen was investigated. To 5 portions of a 1:30 suspension of the gland in saline, sufficient formalin to give concentrations of 0.5, 0.4, 0.3, 0.2, 0.1 per cent was added and the preparations allowed to stand at 4°C. for 3 days. Analogous preparations of normal parotid gland suspension were kept under the same conditions as controls. Titrations were then carried out which indicated that none of these concentrations of formalin appeared to impair the activity of the antigen. Indeed the endpoints in every instance were slightly greater than that found for the material without formalin. This enhancing effect was probably due to a slight anticomplementary action of the formalin.

Attempts were also made to evaluate the effect of 0.5 per cent phenol which has been used as a preservative in material for the skin test (15,21). The suspensions, however, of both normal and infected gland were rendered so anticomplementary by its addition that titrations proved impracticable.

*Filtrability of the Antigen.*—When a 20 per cent suspension of infected gland in saline was passed through an Elford collodion membrane of about 500  $\mu$  A.P.D. under approximately 1 atmosphere positive pressure, complement-fixing antigen was found to be present in the filtrate. Under these circumstances, however, only about one-eighth of the antigen traversed the membrane. It is probable that the inefficiency of filtration was due to the nature of the suspending medium as it is well known that in solutions of sodium chloride various viruses do not pass through filters the pore sizes of which are much larger than the particles of virus. We have not employed broth as a medium since it has been found to exert an anticomplementary effect.

*Deposition of Antigen in the Ultracentrifuge.*—A 20 per cent suspension of infected gland was prepared in phosphate buffer at pH 7.2 and cleared of large particles by centrifugation at 3500 R.P.M. for  $\frac{1}{2}$  hour in the angle centrifuge. Approximately 5 ml. of the supernatant fluid were then rotated in the ultracentrifuge for 1 $\frac{1}{2}$  hours at 220,000  $\times$  gravity. The supernatant fluid was removed in 3 portions of 2 ml., 2 ml., and about 1 ml. The small amount of material in the bottom of the tube was suspended in 4 ml. of phosphate buffer. These specimens were tested for their capacity to fix complement in the presence of mumps antibody. No activity was demonstrated in the upper portions of the supernatant; a slight fixation was observed with the 1 ml. portion. The material recovered from the base of the tube was found to have a titer of the same magnitude as that of the suspension before centrifugation. It can therefore be stated that the complement-fixing antigen is almost completely separated from the suspending medium under the conditions described.

*Failure to Demonstrate Other Serologic Reactions with the Antigen.*—Attempts were made to demonstrate flocculation in the presence of antisera of high titer. In no instance was any indication of a phenomenon of this sort observed. Following the suggestion afforded by certain reports on the agglutination of collodion particles (22) and bacteria (23) to which various viruses were adsorbed, we carried out similar experiments with suspensions of infected gland and homologous antibody. Again the results were entirely negative.

*Failure to Demonstrate Hemoagglutination by the Antigen.*—The peculiar property of certain viruses such as those of influenza (24), fowl plague (25), Newcastle disease of fowls (26), and

vaccinia (27) to cause the agglutination of erythrocytes has recently been described. Accordingly the effect of the mumps antigen on the red cells of a number of species of animals was investigated. The latter included man, monkey, rabbit, sheep, horse, ox, guinea pig, and fowl. No hemoagglutination was observed in any case.

*Failure to Demonstrate Elementary Bodies in Suspensions of Antigen.*—Darkfield examination as well as smears of suspensions of potent antigens stained by the method of Herzberg (28), Giemsa, or Macchiavello's technique (29) have presented no appearances which might be interpreted as elementary bodies.

#### *Properties of the Complement-Fixing Antibody*

In the following account of certain properties of the complement-fixing antibody we have included data derived from the study of the antibody which, as will be shown in subsequent papers, appears regularly in the serum of human beings convalescent from mumps and in that of certain normal individuals. Although we are here concerned with immunologic phenomena of the infected monkey, it seemed most convenient to introduce this material at this point.

*Thermostability.*—The titer of the antibody in monkey serum remains unchanged when the serum is maintained at 62°C. for 20 minutes. When the undiluted serum or dilutions thereof after cooling at ice box temperatures are again heated under these conditions, no significant decrease in the concentration of antibody is observed. This fact has proved of great practical value since it has been found that double heating of a serum which is anticomplementary removes this effect in most instances.<sup>4</sup> It has therefore been possible to use sera in the complement fixation test which have become anticomplementary on storage. The procedure of double heating is also applicable to human sera; but the antibody appears to be less thermostable since heating for 20 minutes at 62°C. appreciably reduces the titer. At 60°C. for 20 minutes, however, the antibody is not affected and the procedure of double heating may be employed under these conditions. When heated at 65°C. for 20 minutes much of the antibody in both human and monkey sera is inactivated.

*Maintenance of Activity during Storage.*—Antisera of both human and simian origin to be employed for the standardization of antigen have been maintained in the frozen state in the CO<sub>2</sub> cabinet; no demonstrable change in titer has occurred even after 6 months. If the means are available, this procedure would seem to be the simplest way to store antisera when it is desired to insure the preservation of the antibody. When it is necessary, however, to keep large numbers of specimens, this method may not be convenient, and recourse must be had to the ordinary ice chest. Comparative studies on 61 human sera kept at 4°C. have shown that the concentration of antibody may decrease rather rapidly. A number exhibited a diminution in titer after 2 months whereas after 8½ months in certain instances in which the initial titer was not high, no antibody could be demonstrated. Fewer data of the same sort are available for monkey sera. Determinations, however, of the antibody before and after storage at 4°C. have shown it is much more stable.

The instability of the mumps complement-fixing antibody in human serum, contrasting with the stability of similar antibodies for other viruses such as influenza, should be borne in mind if specimens submitted to the laboratory for diagnostic test have been preserved for any significant length of time. It is also evident that human sera to be used for the titration of antigens should not be stored in the ice chest but maintained in the frozen state.

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<sup>4</sup> See page 95.

*The Association of the Antibody with Gamma Globulin.*—The quantity of the mumps complement-fixing antibody in specimens of concentrated human globulins prepared by Cohn and his associates from large pools of normal human plasma has been determined (30). The results have shown that in man the antibody is largely associated with the gamma globulin fraction. In this fraction a concentration of the mumps antibody of 20 to 30 times over the plasma has been regularly recorded. A gamma globulin fraction prepared from a pool of convalescent mumps plasma by the method employed for the normal plasma exhibited a concentration of antibody of from 10 to 20 times.

Since certain of the preparations of globulin studied consisted of about 90 to 100 per cent gamma globulin, it is evident that the mumps complement-fixing antibody is closely associated with this serum protein. That it is to be identified with the latter cannot be asserted on the basis of the available evidence, but this hypothesis appears reasonable.

*Failure to Produce Infection or to Demonstrate the Antigen in the Organs of the Monkey Other Than the Parotid and in Certain Tissues of Other Animal Species*

Two male monkeys, 1 an adult, the other a young animal, were inoculated intratesticularly with the virus. The testes were removed on the 5th and 4th days respectively. Saline suspensions of the glands were tested for antigen. None was found although in both animals the parotid glands which had been inoculated at the same time as the testes yielded the usual quantity.

To determine whether antigen might be produced in the brain, a normal monkey was injected into the mid-cerebral area with 0.3 ml. of a 20 per cent suspension of infected parotid gland. Five days later portions of the brain were removed and prepared as antigen according to the method of Casals and Palacios (13). Again no evidence was obtained of the presence of antigen nor was it detected in a sample of the spinal fluid of the same animal.

Employing the method of inoculation *via* the parotid duct, 3 young cats estimated to be about 2 to 4 months old were given 0.5 ml. of 20 per cent infected gland. After 2, 5, and 6 days respectively, emulsions of the glands were tested. No antigenic activity was noted, nor did complement-fixing antibody subsequently develop. These negative findings would appear to afford evidence against the belief that cats are susceptible to the virus of mumps,—a belief established, presumably, by the experiments of Wollstein (31, 32).

A number of rabbits and guinea pigs have been inoculated into the parotid, intratesticularly, intracerebrally, and into the anterior chamber of the eye. No antigen was detected either in the parotid, testes or brain and no lesions developed in the inoculated eye which could be attributed to a specific effect of the virus. Antibody, however developed in certain of those inoculated into the parotid after about 2 weeks.

Virus was injected into the testes of a mature ferret and the animal observed for 3 weeks. No significant rise in temperature nor definite swelling of the inoculated organ were noted. No antibody was found in the serum after 21 days.

An extensive study was carried out in white mice following a result which suggested that the antigen increased in the brain after intracerebral inoculation of virus. Numerous passages by this route were completed but the phenomenon was never observed again nor were symptoms induced which could be interpreted as due to the virus. These negative findings are in agreement with those of Swan and Mawson (33). Antibody was not demonstrated in the sera of mice after inoculation.

Two cotton rats and 1 hamster were inoculated intracerebrally with potent virus. The animals remained well.

A dog was given virus *via* the parotid duct. No swelling of the gland ensued. The organ was not examined for the presence of antigen.

We have also carried out numerous experiments in attempts to propagate the virus in the developing hen's egg. Inoculation has been effected by various routes including the chorioallantoic membrane, the chorioallantoic cavity, the yolk sac, the amniotic sac, and the embryonic brain. Injections have likewise been made intravenously employing the mineral oil method of Brandly (34, 35) as modified by Eichorn (36). Incubation of the inoculated embryos has been maintained at both 35° and 37°C. The egg fluids as well as suspensions of the chorioallantoic membrane, yolk sac, and embryo were tested repeatedly at varying periods following inoculation, but in no instance has it been possible to demonstrate unequivocally the presence of antigen.<sup>5</sup>

Only once has material from the inoculated egg been tested for the presence of virus by the intraparotid inoculation of the monkey. This animal received into the right parotid duct pooled chorioallantoic membrane suspension of the 4th egg passage (37°C.) and into the left duct pooled yolk sac material from the 3rd egg passage (37°C.). Antibody did not develop in the serum as a result of these inoculations. Later, however, following the injection of monkey virus the usual antibody response ensued. This observation is in agreement with most of those of Swan and Mawson (33) who employed 10 monkeys in testing materials derived from developing eggs inoculated with mumps virus. In 2 animals, however, these authors obtained symptoms compatible with the disease but subsequently tests for immunity by challenge inoculation with monkey virus indicated that 1 of these monkeys remained susceptible. It would seem, then, that if the strains of virus which so far have been studied multiply at all in the developing egg, they do so with great irregularity and then only to a very limited degree.

The possibilities of tissue culture were also explored. The roller tube method was employed as adapted to the cultivation of vaccinia virus by Feller, Enders, and Weller (37). Cultures were prepared consisting of (1) chick embryo tissue, (2) various tissues of the monkey such as spleen, testis, parotid gland, and (3) minced human placenta. Chicken serum was incorporated in the nutrient fluid for the embryonic tissue; normal monkey serum for the monkey tissues and monkey or human serum shown to contain no antibody for the placental tissue. After inoculation with the virus the nutrient fluid and tissues were removed from time to time and employed as antigens. In only 1 instance was the antigen demonstrable after the nutrient fluid had been once replenished and that was in cultures which consisted of the infected parotid tissue of the monkey. There, however, merely survival and not multiplication of the virus was demonstrated.

These results, then, illustrate once again the narrow limits of host and tissue range to which viral increase is confined.

#### *Experiments on the Active and Passive Immunization of Monkeys*

*Vaccination with Formolized Antigens.*—The demonstration of considerable quantities of antigen in glandular suspensions together with the development of a means of determining *in vitro* at least 1 type of immunologic response to

<sup>5</sup> In reply to an inquiry as to whether he had been able to obtain evidence for the multiplication of the virus in the developing hen's egg, Dr. Ernest Goodpasture has stated in a letter to one of us (J.F.E.) that he had failed to do so following inoculation of the chorioallantoic membrane. He had not explored the possibility of other routes of inoculation. We are indebted to Dr. Goodpasture for this information.

the presence of the virus induced us to undertake a series of experiments in monkeys designed to show whether or not inactivated virus, when parenterally injected, led (1) to the appearance of antibody and (2) to increased resistance against subsequent intraparotid infection. Vaccines were prepared in 2 ways.

*Formolized vaccine:* Sufficient formalin to yield a final concentration of 0.3 per cent was added to a 10 per cent suspension in saline of infected gland. It was found by injecting the material into the parotid glands of 2 normal monkeys that after standing for 24 hours at 4°C. it did not induce infection as judged by the failure to demonstrate complement-fixing antigen in the inoculated glands on the 5th day. In certain cases this 10 per cent suspension was employed; in others the material was diluted in saline so that the concentration of gland was 2.5 per cent and that of the formalin was 0.075 per cent.

*Alum-precipitated formolized vaccine:* A 20 per cent suspension in saline of infected parotid gland was treated with 0.3 per cent formalin and allowed to stand overnight in the ice box when 0.1 ml. of a 10 per cent solution of potassium alum was added for each milliliter of virus suspension. The material was neutralized with  $\text{NaHCO}_3$  in a concentration of 20 per cent. A white colloidal suspension formed immediately which, after allowing the tube to stand for 1 hour in the ice box, was sedimented by centrifugation at 3500 R.P.M. in the angle centrifuge. After removal of the supernatant fluid, the insoluble material was suspended in a volume of saline equivalent to the original volume. This suspension was used as the vaccine.

The data obtained in 7 experiments in which these vaccines were employed are summarized in Table III. With 2 exceptions, 2 doses of vaccine were given subcutaneously at an interval of 5 days. In 1 instance (M 11) 2 doses of the formolized vaccine were injected intracutaneously and in the other (M 42) only 1 dose of vaccine was administered. For complement fixation tests specimens of serum were obtained before each dose of vaccine was given, at the time of the challenge inoculation of virus, at the time of parotidectomy, and subsequently at various intervals. To test for the development of resistance, active virus was inoculated into 1 or both glands. The content of antigen in the glands was determined by titration on the 5th or 6th day following. The pertinent facts obtained from the study of animals, which did not receive the vaccine but were included in each experiment as controls, are also presented.

It is clear from the combined results that 12 at least of the 18 vaccinated monkeys exhibited moderate titers of complement-fixing antibody at the time of inoculation with active virus. Since it was determined that this antibody was not present in 10 of these animals before vaccination and was probably not present in the other 2, it is likely that in most instances this factor appeared as the result of the injection of the inactivated virus.

This conclusion, however, may not be accepted without qualification, since previous contact with infected animals cannot in every case be excluded which might have led to the development not only of antibody but also to resistance against the challenge inoculation of virus. Such contact is known to have occurred in the case of M 19-39 and is very probable in that of the control, M 27. The latter exhibited no antibody at the time of infection, but 2 days afterwards antibody was found in its serum in a titer of 1:384. This sudden rise so soon after inoculation strongly suggests the previous existence of immunity. The majority of the

TABLE III  
*Immunization of Monkeys with Formolized and Alum-Precipitated Formolized Virus*

Experiment	Monkey	Vaccine dose*	Interval between 1st dose of vaccine and infection	Virus dose‡	Titer complement-fixing antigen in gland§		Titer complement-fixing antibody day of infection	Titer complement-fixing antibody 18 to 30 days after infection
					R	L		
I	19-39	1-10f	28	1-5	<75	ni¶	48**	1536
I	48	—	—	1, 1.5-5	245	175	nd	nd
II	49	1-10f	19	1-5	<75	ni	48	768
II	50	0.3-10f	19	0.5-5	<75	ni	96**	1536
II	1	—	—	0.5, 1-5	150	>225	0	1536
III	9	0.3-10f	15	1, 1-10	75	ni	48	768
III	10	0.3-10f	15	1, 1-10	75	ni	48?‡‡	384
III	11	0.3-10f—ic	15	1, 1-10	213	600	ac**	768
III	12	—	—	1, 1-10	290	290	0	768
IV	23	0.5-10f	14	1, 1-2.5	150	425	0	24
IV	24	0.5-10af	14	1, 1-2.5	213	213	96	384
IV	25	0.5-2.5f	14	1, 1-2.5	53	<38	0§§	384
IV	26	0.5-2.5af	14	1, 1-2.5	<38	<38	96	384
IV	27	—	—	1, 1-2.5	<38	<38	0	1536
V	29	0.5-10af	14	1, 1-2.5	150	150	48	384
V	30	0.5-2.5f	14	1, 1-2.5	150	105	0	nd
V	31	0.5-2.5af	14	1, 1-2.5	<75	<75	24	384
V	32	—	—	1, 1-2.5	290	213	0	nd
VI	34	0.5-10f	14	1, 1-2.5	150	150	24	nd
VI	33	—	—	1, 1-2.5	600	600	0	nd
VII	37	0.5-2.5af	21	1, 1-1.3	150	75	0	384
VII	38	0.5-2.5af	21	1, 1-1.3	75	75	48	1536
VII	40	0.5-2.5af	21	1, 1-1.3	<38	<38	24	1536
VII	42	0.5-2.5af¶¶	15	1, 1-1.3	>150	nd¶	96	736
VII	35	—	—	1, 1-1.3	290	290	0	1536

\* The first figure indicates the volume of each dose in milliliters of vaccine; the second figure refers to the percentage concentration of infected parotid gland; "f" denotes vaccine containing 0.3 per cent formalin; "af" denotes vaccine prepared by precipitating the formolized material with potassium alum; vaccine was given by the subcutaneous route except in the case of M 11 in which it was inoculated intracutaneously. With the exception of M 42 which received 1 dose of vaccine, each of the other animals received 2 doses at an interval of 5 days.

‡ The first and second figures give the volumes of inoculum in milliliters of the right and left gland respectively. The third figure indicates the percentage concentration of infected parotid.

§ Reciprocals of the highest dilution of gland suspension causing complete fixation of complement in the presence of excess antibody.

|| Reciprocals of the highest dilution of serum giving + or ++ fixation.

¶ ni = "not inoculated"; nd = "not done."

\*\* Specimens of serum secured before the first dose of vaccine were somewhat anticomplementary or fixed complement in the presence of normal parotid suspension. Therefore, although it is probable that antibody developed as a result of vaccination, it is impossible to be certain.

‡‡ This titer was obtained with serum drawn the day following inoculation. No antibody was detected in the specimen taken the day of inoculation.

§§ 2 days before inoculation the titer of the serum was 48. It is difficult to account for the sudden disappearance of antibody.

||| Although no antibody was demonstrated on the day of infection, 2 days later the titer was >384. This observation strongly suggests that the animal had previously been exposed to the virus.

¶¶ Monkey 42 received only a single dose of vaccine.

animals were kept in quarters which were remote from those of infected monkeys until the time they were employed in these experiments. Accordingly we are reasonably certain that in most of them previous inapparent infection was not responsible for the results. Further evidence of the capacity of such vaccines to stimulate the formation of antibody is afforded by the results of experiments in human beings which will be described in a later communication (16).

Since in none of the animals was antibody demonstrated 5 days after the first dose of vaccine, it might be inferred that a single injection represented an inadequate antigenic stimulus. It is possible, however, that this interval is too short to permit antibody formation. Thus it is of significance that M 15, which received only 1 dose of vaccine, was found to have developed antibody by the 15th postvaccinal day.

In determining whether or not resistance to intraparotid inoculation of virus was induced as a result of vaccination, we have depended upon demonstrating on the 5th or 6th day the absence or presence of complement-fixing antigen in the gland. Because the antigen appears regularly in susceptible animals which have received quantities of virus comparable to those employed for challenge inoculation we believe, as previously stated, that its absence is a fairly dependable criterion of resistance. It has also been shown that convalescent monkeys challenged with active virus fail to develop antigen in the inoculated gland. Moreover it seems reasonable to regard titers of antigen which are definitely lower than those usually encountered in susceptible monkeys as suggestive evidence for some degree of resistance. Parotidectomy might have been postponed until after the expected period of symptoms thus affording another criterion for estimating the effect of vaccination. This was not done because at that time antigen may have disappeared from the gland. Moreover, as already stated, the manifestations of parotitis may often be minimal and difficult to recognize.

With these considerations in mind, we may proceed to an examination of the data included in columns 6 and 7 of Table III.

If the titers of antigen in the glands obtained from the vaccinated animals be compared with those derived from the controls, it is found that the frequency of low values is significantly greater in the former. Thus in 10 among 16 of the animals which received 2 subcutaneous doses of vaccine, the glands showed antigen titers of 1:75 or less. In 5 individuals no antigen could be detected in the lowest dilution of suspension it was possible to test without encountering anticomplementary effects; in 2 other monkeys doubtful fixation was obtained with 10 per cent suspensions of the glands. On the other hand, among 7 unvaccinated controls, 1 only failed to produce considerable quantities of antigen. This exception (M 27) gave evidence of having had a previous encounter with the virus. If, instead of the number of animals, the number of glands with titers of 75 or below are considered, it is found that of 27 glands from the vaccinated group, 17 contained little or no antigen whereas it was lacking in only 2 among 14 of the glands derived from the controls. In Table IV these data are further analyzed. To the results obtained with the unvaccinated monkeys mentioned in Table III, are added the titers of the glands from 21 other animals which have been presented in Table I.

It is evident that whether the analysis is made on the basis of the numbers of individuals or of glands, the conclusions which may be drawn are the same. Thus approximately 65 per cent of the vaccinated animals yielded glands in which little or no antigen could be discerned whereas in about the same proportion of unvaccinated monkeys the titers of antigen exceeded 1:150. In about 25 to 30 per cent of both groups a moderate amount of antigen (titer 1:150) was demonstrated. Although the numbers, especially in the vaccinated group, are small and although the mode of preparation of the vaccines and their dosage varied as well as the quantity of virus, it would seem reasonable to attribute these differences mainly to a prophylactic effect of the vaccination.

TABLE IV  
*Complement-Fixing Antigen in Glands of Normal and Vaccinated Monkeys*

Monkeys	Monkeys with one or both glands having titer of								Total No.
	<75		75		150*		>150		
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	
Normal.....	1	3.5	1	3.5	9	31	18	62	29
Vaccinated.....	7	43.8	3	18.7	4	25	2	12.5	16
Glands from monkeys	Glands having titer of								Total No.
	<75		75		150†		>150		
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	
Normal.....	2	4.1	3	6.1	12	24.6	32	65	49
Vaccinated.....	11	40.8	5	18.5	8	29.6	3	11.1	27

\* Monkeys yielding glands with titers between 75 and 150 are included.

† Glands with titers between 75 and 150 are included.

But it is equally manifest that none of the procedures was universally effective. Apparently the response of the individual animal to the antigen varied considerably and it is this variation of the host rather than the differences in the quantities of vaccine or its mode of preparation which seems best to account for the lack of uniformity in the degree of resistance which is recorded.

A comparison of the quantity of antigen in the gland and the titer of antibody in the serum at the time the virus was inoculated (Table III, columns 6, 7, and 8) fails to reveal a uniform inverse relationship between these two reagents. It is, therefore, impossible to affirm that the antibody concerned in complement fixation is by itself responsible for protection. Accordingly we must assume that another factor or factors are in part, at least, responsible for the enhanced resistance.

The same comparison, however, shows that the antibody was present at the time of the challenge inoculation in 8 of the 10 vaccinated animals which had little or no detectable antigen in their glands. In 1 of the 2 exceptions (M 25), antibody had been found 2 days before and in the other (M 37) 1 gland had a moderate quantity of antigen, suggesting only a slight degree of resistance. It would seem, then, that although the antibody cannot be necessarily correlated with resistance, it was usually present in those animals which proved more or less refractory to the inoculation of the virus. Therefore this serum factor cannot be entirely eliminated as a participant in resistance.

It will be observed from inspection of column 9 of Table III that in nearly all the vaccinated monkeys the quantities of complement-fixing antibody following the challenge inoculation of virus increased to levels comparable to those of the unvaccinated animals.

Two possible explanations for this phenomenon present themselves. The inoculation of virus may simply act as a third antigenic stimulus which might lead temporarily to the further increases in antibody. That the introduction of inactivated virus into the parotid gland of normal monkeys can give rise to the formation of antibody has been established in experiments which are not here recorded in detail. On the other hand, the increase may be dependent upon the establishment of a mild atypical infection in which multiplication of virus is slight but which is sufficient to raise the antibody level to that of the ordinary convalescent. It would be difficult to obtain direct evidence for the validity of this conception. If it were found that antibody were demonstrable for many months in the serum of such animals, one would be inclined to regard its persistence as the result of infection and not that of simple antigenic stimulation. Since we have made no determination of antibody later than 3 months after the inoculation of vaccinated monkeys, such evidence is not available. It can be stated, nevertheless, that at this time antibody was still present.

In spite of the fact that only about two-thirds of the vaccinated animals gave evidence of resistance to intraparotid inoculation of the virus, there is reason to believe that the use of vaccine as a means of prophylaxis in human beings might be effective. Not only have tests in human subjects (16) already afforded a certain amount of direct support for this belief, but it must be remembered that the experimental conditions in the case of the monkeys would seem to be such as to provide a much more rigorous trial of resistance than that which would occur under natural conditions of infection. Thus considerable quantities of virus have been brought directly in contact with the cells of the parotid gland in a manner which would seem largely to preclude the participation of any protective factors which may exist in the serum of these animals.

*Passive Immunization with Human Convalescent Mumps Serum and Globulin Concentrate.*—Johnson and Goodpasture (6) were unable to demonstrate unequivocally any protective or neutralizing properties in the convalescent serum of man or monkey employing the latter as the experimental animal. The techniques described in this communication seemed to furnish the means of securing further evidence in respect to the problem. Two experiments have

been carried out. In one pooled human convalescent mumps serum was employed and in the other the globulin concentrate prepared by E. J. Cohn and his collaborators from the same pooled convalescent serum (30). In both experiments the serum or globulin was mixed with a suspension of virus and inoculated into the parotid glands of monkeys. The details of procedure and the results are given in the following protocols.

*Protocol 1.—The Effect of Human Convalescent Mumps Serum*

The pool consisted of sera from 177 individuals convalescent from mumps and collected at Camp McCoy by Major Aims C. McGuinness, of the Commission on Measles and Mumps, during the winter of 1943. Its complement-fixing titer was between 1:192 and 1:384. The virus consisted of a saline suspension of the parotid gland from the 10th monkey passage. Mixtures of equal parts of virus and undiluted serum were prepared as noted below and allowed to stand at about 27°C. for  $\frac{1}{2}$  hour. Each monkey was given 1 ml. of mixture or of virus alone into the right parotid. The left gland was not inoculated. Before inoculation no antibody was found in the monkeys' sera.

Both glands were removed from each animal 6 days later when definite enlargement of the right gland was noted in all except M 21. In each the amount of antigen was determined. None was demonstrated in the glands which had not been inoculated. The results of the titrations of the inoculated glands were as follows:—

Monkey No.	Inoculum	Titer of antigen in right parotid gland
18	Virus diluted 1:80 and serum	52
19	“ “ 1:40 “ “	150
20	“ “ 1:20 “ “	75
21	“ “ 1:40. No serum	1200
22	“ “ 1:160 “ “	600

Clearly the quantity of antigen in the glands of the monkeys which had received mixtures of serum and virus was less than that found in both of the animals which received virus only. In none of the 3 monkeys, however, in which the serum appears to have exerted an inhibitory effect on the increase of the antigen was its formation entirely suppressed.

It is obvious, of course, that to determine by means of the technique we have employed whether or not such serum possesses true virus-neutralizing properties, it would be essential to ascertain the minimal infecting dose of virus. Since we are ignorant of it, the doses of virus which we have used in this and the following experiment may possibly exceed any neutralizing capacity of the serum.

*Protocol 2.—The Effect of the Globulin Concentrate*

The globulin concentrate was prepared from the pool already mentioned. Its titer of antibody was between 1:1800 and 1:3600. A mixture of equal parts of concentrate and a 1:20 suspension of a gland of the 11th monkey passage was prepared, allowed to stand at room temperature (*i.e.* about 21°C.) for 1 hour. One ml. of the mixture was inoculated into each duct of 2 monkeys. As controls 2 other monkeys were also inoculated with virus only.

Antibody was not demonstrated in the serum of any of the monkeys before inoculation. On the 6th day swelling of both glands of the controls was apparent. No definite glandular enlargement was observed in the animals which received the mixtures. The glands of all the monkeys were removed and, with one exception, their content of antigen determined. The results were as follows:—

Monkey No.	Inoculum	Titer of antigen in gland	
		RP*	LP†
62	Virus diluted 1:20 and globulin	<75	<75
20-58	“ “ “ “ “	75	75
20-49	“ “ 1:40	105	130
20-56	“ “ “	600	—

\* Right parotid. † Left parotid.

As with the convalescent serum, there would appear to be a definite inhibition by the concentrate upon the increase of the antigen, but again complete suppression of viral activity was not obtained in the case of M 20-58, although in M 62 no antigen could be shown.

These findings strongly imply that it is not, as suggested above, the lack of sufficient antibody to neutralize the virus which is responsible for the failure to obtain complete suppression of viral multiplication as reflected by the increase of antigen; more probably either an efficient neutralizing factor is absent or the conditions of the experiment are such as to preclude in most instances its effective operation.

The final solution of this problem would seem to depend on the results of controlled tests in human beings in whom mumps can be produced by spraying the virus into the oral cavity, since it is not unlikely that circulating antibody may be much more effective in interfering with the pathogenesis of the natural disease than in that of the infection induced by direct inoculation of the susceptible tissue.

#### SUMMARY

Since the experimental data have already been discussed at some length, it is here necessary only to review those observations which appear to be of most significance.

The manifestations of the disease we have produced in monkeys have in general been similar to those described by previous workers. It has been found that members of the species *M. mulatta* are regularly susceptible to infection with the virus of mumps, provided they have not been in contact with animals which have recently been infected. Such contact may be followed by the development of resistance to inoculation unattended by overt signs of infection.

Following the intraparotid injection of virus into a susceptible animal, an antigen appears in the gland which reacts specifically in the complement fixation test with sera of monkeys which have recovered from an attack of

mumps. This antigen has not been demonstrated in any other organ of the infected monkey which has been examined, nor does it persist or increase in amount when introduced into the brain or the testes. Attempts to propagate it in the tissues of other animal species including the developing hen's egg and tissue cultures have so far been uniformly unsuccessful. The antigen is relatively thermostable, resisting a temperature of 65°C. for at least 20 minutes at the optimal hydrogen ion concentration. It is unimpaired by a concentration of formalin of 0.3 per cent. Together with the virus it may be preserved indefinitely in the frozen state. The maximal quantity of antigen in the gland, which may be considerable, is attained on or about the 5th day following inoculation of the virus.

An antibody which reacts with the antigen appears in the serum from 8 to 14 days after infection. This antibody has not been found in the serum of normal monkeys which have had no opportunity for contact with the virus. It has been demonstrated during convalescence in the serum of all animals which have been examined. The antibody which is present in large amounts soon after recovery, although decreasing in concentration with the lapse of time, may continue to be present for many months. At 62°C. for 20 minutes its activity is not reduced and on storage at 4°C. its titer has remained unaltered for over 2 years. A similar factor which appears in human beings convalescent from mumps is less stable, since its activity is reduced appreciably under these conditions. In the CO<sub>2</sub> cabinet, however, both antibodies can be preserved for long periods of time.

The definition of these reagents has afforded means whereby the simian infection with the virus of mumps, whether it is actively in progress or has occurred at some time in the past, can be recognized.

Upon the establishment of these facts, the estimation of the quantity of antigen in the glands of vaccinated animals was employed as an index of their resistance in studies on the effect of the injection of formolized and alum-precipitated formolized suspensions of infected parotid gland. It has been shown that such vaccines, in which the virus has been rendered inactive, lead to the production of complement-fixing antibody. In about 60 per cent of the vaccinated animals, moreover, evidence of increased resistance was obtained as indicated by the partial or complete suppression of the formation of antigen in the inoculated gland. In certain instances no evidence of resistance was observed, yet antibody was found to be present. It is clear, therefore, that resistance is not determined solely by the presence of the complement-fixing antibody. The possibility, however, of its participation as a factor in immunity cannot be excluded.

Employing the same criterion of resistance, the virus-neutralizing capacity of a pool of human mumps convalescent sera and of a globulin concentrate prepared from the pool has been studied. Neither of these materials was found

to prevent entirely the formation of antigen, although each of them appeared to exert some inhibitory effect.

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