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APPENDIX

# INFLUENZAL ANTIBODIES IN LYMPHOCYTES OF RABBITS FOLLOWING THE LOCAL INJECTION OF VIRUS

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## INTRODUCTION

In recent years, a series of developments has pointed to the role of the lymphatic system in the formation of antibodies. Early investigations (1–3) indicated that following introduction of an antigen into the tissues of an animal, antibodies could be found in the regional lymphnode, often appearing there earlier than in the blood-serum. More recently, two series of studies have been concerned with the relation of the lymphatic system to the production of antibodies. In one of these, that of White and Dougherty, it was shown that preparations of the spleen and lymphnodes rich in lymphocytes contained antibodies following subcutaneous injections of antigen into mice (4, 5). In the other series of studies Ehrich and Harris made use of the fact that the popliteal lymphnode of the rabbit is the sole node draining all tissue distal to it. Cellular antigens were injected into the pad of the rabbit's hind foot, and simultaneous studies were made of extracts of the popliteal lymphnode, the lymph of the afferent and efferent lymph-vessels of that node and the blood-serum. These investigations showed that the lymphnode and efferent lymph contained antibody in such concentration, and so soon after the injection of antigen, as to indicate clearly some role of the lymphnodes in the formation of antibodies (6). On further analysis (7), lymph was separated by centrifugation into lymph-plasma and lymphocytes. Examination of each of these separately showed that the lymphocytes contained antibody in higher concentration than the lymph-supernate. Cross-absorption studies further pointed to the fact that the lymphocytes had not absorbed the antibodies from the lymph-plasma but were the primary site of these substances.

The immunological findings were correlated with histological changes taking place in the local lymphatic tissue. In other studies of this series it was shown that macrophages did not, on contact with antigens *in vivo*, produce antibodies (8), and the fate of particulate antigens was traced from the time of injection until that of the appearance of antibodies (9).

Since the studies referred to above involved the injection of bacterial or cellular antigens and whole cells, interest was aroused as to whether a similar mechanism might operate in the production of antibodies to other antigens such as viral proteins. Two of the earlier studies mentioned had been concerned with the sequence of events following injection of active virus. McMaster and Kidd (2) had demonstrated an antiviral principle in extracts of regional lymphnodes following the endermal injection of active vaccine-virus into the ears of rabbits. The neutralizing principle was found in higher concentration in the lymphnode

than in the serum during the first week of the experiment. Evidence of multiplication of the virus introduced was found in the lymphnodes until the appearance of antibody. Burnet and Lush (3), infected mice with virulent influenzal virus via the intranasal route, and found antibody to influenzal virus in the mediastinal lymphnodes in 4 to 6 days.

In the present study it was felt desirable to investigate the development of antibodies to a viral agent, employing the agent as an antigen, with no possibility of multiplication of the virus in the tissues. Accordingly, a study was undertaken of the immunological response in the rabbit to the injection of preparations of influenzal virus, inactivated by exposure to ultraviolet rays, utilising conditions similar to those prevailing in the experiments quoted above with bacterial and particulate antigens (6–9). The preparations of inactivated influenzal virus were injected into the foot-pad of the rabbit and at various intervals thereafter the following materials collected: lymph from the efferent lymphatic vessel of the popliteal lymphnode, the node itself and heart-blood. In one series of experiments, one type of influenzal virus was injected into one foot-pad, and a heterologous type was injected into the other foot-pad. This provided for a further control on the specificity of the reaction.

## METHODS AND MATERIALS

*Preparation of Viruses.*—The preparations of the PR8 strain of influenza A and the Lee strain of influenza B viruses were made by inoculating 10-day-old chick-embryos with 0.2 ml of a $10^{-4}$ dilution of the respective seed-cultures.* The eggs were then further incubated at 37 C for 48 hours, after which the allantoic fluids were harvested aseptically. The fluids to be used as vaccines were centrifuged for 20 minutes at 20,000 rpm in a high-speed centrifuge, the supernatant fluid was discarded and the sediments resuspended in sterile buffered physiological saline solution in $\frac{1}{10}$ of the original volume. The concentrated virus was inactivated by exposure to ultraviolet rays for 10 minutes (10). All preparations were tested for their capacity to agglutinate chicken-erythrocytes and those vaccines which were used had similar titers. Of these vaccines, 0.2 ml was injected into the hind foot-pad of rabbits. The allantoic fluids injected with influenzal virus which were to be used as antigen in serological tests were dialyzed against 20 volumes of buffered saline in order to remove urates and to prevent formation of precipitates on storage at 4 C.

*Injection of Rabbits.*—The rabbits injected were female albinos or chinchillas weighing generally about 2000 g. Prior to any injection, each rabbit was bled from the heart, the serum collected and preserved to be tested with later specimens. The foot-pads of the rabbits were shaved and injected with 0.2 ml of the antigen-preparation and the point of entrance of the needle sealed with a drop of collodion. After suitable periods of time the rabbit was anaesthetized, the skin of the inner aspect of the knee was incised, the *semitendinosus* and *semimembranosus* muscles cut, and the popliteal lymphnode exposed. A ligature was

* A preparation of the PR8 strain of influenza A virus and the Lee strain of influenza B virus were kindly supplied by Dr. Werner Henle.

placed around the efferent lymphatic vessel and lymph was collected through a 27 gauge needle into a syringe moistened with a solution of sodium citrate. The lymphnode was then excised and blood was collected from the heart.

The lymph was mixed well, enough was drawn off for a white-blood-cell count, and the remainder immediately centrifuged to separate cells from plasma. Each part was frozen and stored at −10 C until tested. The lymphnode was weighed and ground in a mortar with equal volumes of "alundum" and normal saline solution. After grinding, sufficient saline was added to make a 1:16 dilution of the contents of the lymphnode. The ground suspension was cleared by centrifu-

TABLE I

*Relative Concentrations of Antibody in Lymphocyte-extract and Lymph-supernate*

| RABBIT NO. | LEG | INOC. WITH | DAYS AFTER INOCULATION | LYMPH | | | | | | RATIO OF TITER OF CELLS TO SUPERNATE |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Volume collected | White blood cell count per mm.³ | Volume of lymphocytes | Titer of | | | |
| | | | | | | | Lymph supernate | Lymph supernate 1/10 vol. | Lymphocyte-extract | |
| | | | | ml. | | ml. | | | | |
| 34 | L | PR8 2x* | 3 | 0.25 | 75,800 | .0038 | 256 | 256 | 4096 | 16 |
| 39 | L | PR8 20x | 3 | 0.25 | 38,650 | .0019 | 1024 | 1024 | 8192 | 8 |
| 40 | L | PR8 20x | 3 | 0.25 | 26,900 | .0014 | 512 | 512 | 8192 | 16 |
| 40 | R | PR8 20x | 3 | 0.30 | 30,500 | .0018 | 512 | 512 | 6072 | 12 |
| 36 | L | PR8 2x | 5 | 0.35 | 24,400 | .0017 | 512 | 512 | 2048 | 4 |
| 36 | R | PR8 2x | 5 | 0.30 | 48,100 | .0029 | 512 | 512 | 4096 | 8 |
| 42 | R | PR8 20x | 5 | 0.25 | 31,700 | .0016 | 512 | 1024 | 2048 | 2 |
| 42 | L | PR8 20x | 5 | 0.30 | 39,050 | .0023 | 512 | 768 | 2048 | 3 |
| 38 | L | PR8 2x | 7 | 0.20 | 29,600 | .0012 | 512 | 512 | 1024 | 2 |
| 62 | L | Typhoid | 2 | 0.55 | 26,000 | .0029 | <64 | <64 | <64 | |
| 65 | L | Typhoid | 3 | 0.65 | 37,000 | .0048 | <64 | <64 | <64 | |

* These preparations of influenzal virus were concentrated either 2-fold or 20-fold, as indicated.

gation, and the supernate was removed, frozen, and stored at −10 C until tested. Serum was prepared from the heart-blood and similarly stored at −10 C.

*Lymphocyte-extracts.*—The volume of lymphocytes obtained by centrifugation of efferent lymph was obtained by an expression—cell-volume = 0.0002 $T$ $V$ ml where $T$ equals the total cell-count of the lymph in thousands, and $V$ the volume of lymph collected. This expression was derived in an earlier study (7) and results obtained with it were correlated with volumes derived experimentally by hematocrit-determinations. Such total lymph-cell volumes are shown in table I. In preparing the extract, a volume of saline solution 127 times that calculated for the lymph-cells was added to the cell-sediment, and the cells were dispersed in the suspending medium. This suspension was subjected to alternate freezing and thawing, at −70 C and 30 C respectively, three times, and the suspension

was then cleared of insoluble material by centrifugation. The resulting supernate was used as 1:128 solution of the contents of the cells.

*Technic of Antibody-determination.*—Two convenient methods were available to test specimens for content of influenzal antibodies. Both methods utilise the phenomenon of agglutination of chicken-erythrocytes by allantoic fluids infected with influenzal virus (11), and the corresponding inhibition of this agglutination by antibodies to influenzal virus. The original method described by Hirst and Pickels (12) involves the use of a photoelectrical cell to measure the degree of sedimentation of erythrocytes agglutinated by influenzal virus, and the inhibition of this agglutination in the presence of immune bodies. Salk (13) and others have modified this method so that the pattern formed on the bottom of a test-tube by the settling of agglutinated erythrocytes can be used for similar determinations. Because of the somewhat greater sensitivity, relative ease and simplicity of the Salk modification this technic was used in very early experiments. It was found, however, that the greater sensitivity of the pattern-method held no advantage over the Hirst technic since the amount of non-specific inhibition of agglutination by normal-tissue factors was also greater in the pattern-test. Accordingly, all determinations were made by the method originally described by Hirst and Pickels (12), except in a few instances where the volume of material to be tested was insufficient. Serial dilutions of extracts of lymphnode, blood-serum and lymph were made in steps of two. All dilutions were begun at 1:16, as it was found that serum and tissue-extracts of normal rabbits showed some inhibition of the agglutination of chicken-erythrocytes in lower dilutions. One half ml of a suitably diluted antigen was added to 0.5 ml of each dilution of extract of lymphnode, lymph, and serum, respectively, and the mixture was incubated at room-temperature for 10 minutes. Thereafter, 1 ml of a standardized 1.5 per cent suspension of fresh red blood cells was added by automatic pipet. After 75 minutes at room-temperature the degree of sedimentation of agglutinated red blood cells was determined by use of a photoelecrical cell. The end-point was considered to be the last dilution of serum showing inhibition of agglutination to such an extent that between 50 and 63 per cent of the red blood-cells were left in suspension. Although it was possible to render the test quite sensitive by diminishing the concentration of virus to be used, it was found that the inhibitory effects of sera and tissue-extracts of normal animals (11, 14, 15) on the agglutination by influenzal virus were more marked as the test was made increasingly sensitive. With 8 units of virus, as was used in the tests, non-specific reactions were not given by lymphnode-extracts at a dilution of 1:16 or greater. Accordingly, this was the minimal dilution employed in the tests for antibodies. In each test a number of specimens from previous tests were repeated and standard antisera were included, so that correlation could be made from one test to the next.

In the case of the lymphocytes, the small volumes of cell-extracts precluded the possibility of employing the sedimentation-test. It was found, however, that the antibody-titers in the contents of the lymphocytes were so high that the pattern-test could be employed, since the range of titers involved in the test was

beyond that at which non-specific interference of tissue-extracts and fluids occurred. Since the point at issue was whether the lymphocytes were richer in antibody than the lymph-plasma surrounding them, the pattern-test was used only for lymph-specimens of which the lymph-cell sediment was sufficient for a microserological test.

The pattern-test for lymph-specimens was set up by adding 0.4 ml of the antigen in suitable dilution to 0.4 ml of serial dilutions of lymph. After 10 minutes of incubation at room-temperature 0.2 ml of a 1 per cent suspension of cells was added, and the test was incubated at 4 C until the red cells had settled to the bottom of the test-tubes. The inverse of the last dilution of antibody-preparation showing complete inhibition of agglutination of the red cells by influenzal virus was considered to be the titer. In each test antibody determinations were made on specimens that had been previously tested by the use of the photoelectrical densitometer. The ratio of titers obtained in the pattern-test to titers obtained in the sedimentative test enabled us to transfer from one system to the other.

For testing the lymphocyte-extract the volumes of the reagents were reduced tenfold, without altering the respective concentrations or proportions. Thus, the twofold dilutions of cell-extract and the influenzal virus were present in 0.04 ml. All these quantities were delivered from pipets graduated in 0.001 ml, and tubes of 10 mm diameter were employed. It was found that the test could be carried out by this micro-method, and that the titers of standard specimens, examined simultaneously by the regular and micro-tests, showed excellent agreement.

## EXPERIMENTAL

### *Preliminary Observations*

In the first series of experiments undertaken, a concentrate of a commercially prepared vaccine of influenzal viruses of types A and B was used. This preparation consisted of allantoic fluid infected with the PR8 and Weiss strains of type A and Lee strain of type B influenzal virus concentrated 200-fold by centrifugation and inactivated by the addition of 0.05 per cent formalin. When the popliteal lymphnodes were excised 3 days following injection of the vaccine, gross examination showed that the nodes were very large, hemorrhagic, and intensely swollen. The same gross picture characterized the popliteal lymphnodes excised on the 5th day following injection of the vaccine. Of the rabbits included in the group to be sacrificed on the 5th and 8th days after injection, some were found to have developed sterile abscesses at the site of injection on the foot-pad. Histological examination of these lymphnodes showed severe destruction of lymphocytes and of the architecture of the nodes. Karyorrhexis was marked, with bits of nuclear contents replacing lymphocytes. Outlines of the follicular architecture could barely be discerned. In view of the experience gathered with the lymphocytopenic effect of influenzal virus preparations when injected intravenously into the rabbit (16), it was felt that perhaps the toxic effect on the local lymphatic tissue was due to this property of the particular viral agent employed.

Despite the extensive damage to the lymphnodes, analysis of the extracts showed antibody to influenzal virus was present. Similarly, the blood collected from the rabbits' hearts contained antibody to influenzal virus whereas serum collected prior to injection showed no reaction.

*The Optimal Concentrations of Antigens*

Further experiments were undertaken with preparations of the PR8 strain of influenza type A cultivated for the purpose in this laboratory. Various preparations were injected into rabbits' feet, ranging from 20-fold concentrations of virus to $10^{-4}$ dilution with respect to the concentration found in allantoic fluid. No demonstrable antibodies to influenzal virus were found in lymphnodes excised from rabbits which had been injected with 0.2 ml of influenzal virus diluted to $10^{-4}$. However, following the injection of allantoic fluid infected with influenzal virus, at a dilution of 1:100 or less, antibodies could be found in the local lymphatic system. Undiluted allantoic fluid used as antigen produced almost a maximal antibody-response as compared with that to higher concentrations of virus, whereas dilution of the allantoic fluid as antigen caused the appearance of progressively smaller amounts of antibody. Antibodies to influenzal virus appeared in the lymphnode from two to four days after injection of the virus into the foot-pad, whereas normal lymphnodes or lymphnodes derived from rabbits injected with typhoid or dysentery bacilli showed no reaction with influenzal virus. If antibodies to influenzal virus were detected in the serum at about the same time as in the lymphnode they were generally found in lower concentration. On other occasions antibody to influenzal virus was found in the serum 1 or 2 days after its appearance in the lymphnode. The experiments indicated that it was desirable to use an adequate, but not overwhelming amount of virus in the vaccine. A 10-fold concentrate of allantoic fluids which had been infected with influenzal virus and harvested after 48 hours of incubation seemed to be the optimal type of vaccine, although the resulting antibody-titers were not much greater than those that followed the injection of allantoic fluid. Accordingly, all subsequent experiments were conducted with this concentration of influenzal virus.

*Sequence of Events Following Injection of the Viral Antigens*

A series of experiments was undertaken using as antigen an inactivated preparation of the PR8 strain of influenzal virus which had been concentrated 10 times as judged by the capacity of the vaccine to agglutinate chickens' red cells. The antigen was injected into the foot-pads of a suitable number of rabbits and at 1, 2, 3, 4, 5, 7, 9, 10, and 15 days after injection, lymph was collected from the efferent lymph-vessels, the popliteal lymphnode was excised and blood was collected from the heart. Counts of the white blood-cells contained in the lymph collected were found to range from 33,000 to 110,000 cells per $mm^3$, in the period of the 2d to 4th day after injection. By the 9th day, the counts were down to 10,000 to 15,000 and even somewhat lower by the 16th day. The counts found in the later days resemble those which had been found to be characteristic of

lymph collected from the efferent lymph-vessel of the popliteal lymphnode in normal rabbits (6). When the extracts of lymphnodes were tested, it was found that antibody could not usually be detected before the second day following injection of the antigen. On that day, antibody could generally be found in the extracts of lymphnodes in low titer. In subsequent days the level of antibody-titer rose and generally reached the peak between the 5th and 7th day. Frequently a fall in antibody-titer of the extracts of lymphnodes could be detected after the 5th or 7th day. Analysis of the lymph collected at the various intervals similarly showed no measurable amount of antibody before the 2d day, low titers of antibody on the 2d or 3d day, and an increasing titer in later days. Simultaneous tests with the blood-serum showed that no measurable amount of anti-

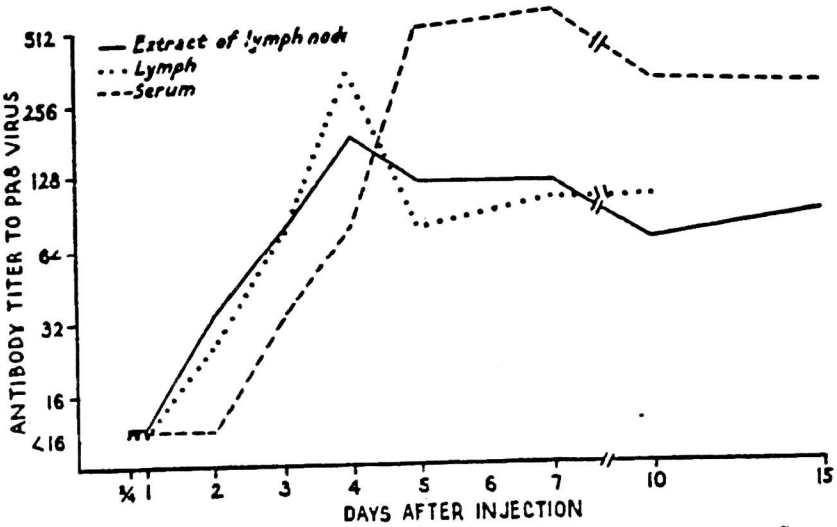


FIG. 1. GEOMETRIC MEAN ANTIBODY-TITERS OF LYMPH, LYMPHNODE-EXTRACT AND SERUM AT VARIOUS INTERVALS FOLLOWING THE INJECTION OF INACTIVATED INFLUENZAL VIRUS INTO THE FOOT-PAD

Each point represents 9 rabbits.

body was present usually before the 3d day. Here again, after the appearance of antibodies in low titer there was a continuous rise. The serum-titer in the first 4 days almost always lagged behind the antibody-titers of both the lymphnodes and lymphs of the corresponding days. By the 5th and 7th days the antibody-content of the serum was greater than that of either the respective lymphnode or the lymph. Thereafter, the serum-titer remained higher for the duration of the experiments. These quantitative relations are shown in fig. 1, which represents a summary of experiments performed with the PR8 strain of influenzal virus, type A. The geometric mean antibody-titer was determined for an average of nine rabbits per interval represented. In the case of the lymph, this number of specimens was smaller because it was not in each case possible to obtain a satisfactory specimen of lymph. Analysis of the results obtained from

rabbits where lymph, extracts of lymphnode and serum were obtained showed that frequently the antibody-content of lymph collected in the 2d to 4th days was higher than that of the lymphnode or serum. In some animals the differences were quite marked, in others, the differences were small, and in some, this difference was not apparent. In all of these cases the antibody-titer of the lymphnode-extract was higher than that of the corresponding serum. In those instances where the titer of lymph was not markedly higher than that of the lymphnode the titer of the extract of lymphnode was nevertheless higher than that of the corresponding serum.

The range of individual variation among the experimental animals is illustrated in table II. On examination of this table it is seen that specimens from rabbits 328, 317 and 341 showed greater differences between the antibody-content of lymph, lymphnode and serum than did the specimens from rabbits 330 and 316. Rabbit 340 illustrates an instance where antibody-content of lymph and lymphnode were similar, and in rabbit 214 the titer of lymphnode-extract was greater than that of the lymph collected.

*Histological Changes in the Lymphnode*

The weight of the popliteal lymphnodes increased progressively with time after injection, from a normal of 0.2 g in the uninjected leg, to weights of 0.7 to 0.8 g. This peak was attained at the 5th to 7th day, and after 10 days, the weight of the lymphnodes began to decline. At about the 4th or 5th day, the entire surface of the node showed very fine irregularities, the external evidence of follicular structure within.

Microscopically there was marked diffuse hyperplasia of lymphoid tissue reaching a maximum two days after the injection. The enlargement of the node was seen to be due to swelling of the cortex with great numbers of large and medium lymphocytes, which were not fitted into any units of organization. Mitotic figures were often seen, and transitional forms between reticulum-cells and the young lymphocytes mentioned above. On the third day there was further increase in size and a number of small lymphocytes were seen. On this day there were beginnings of groupings of small lymphocytes into circular areas. On the fourth day these areas were clearly recognizable as secondary nodules, and by the fifth day, the larger part of the cortex consisted of these clearly defined nodules and many of the lymphocytes were of the small type. At this time large lymphocytes, some reticulum-cells and transitional forms were to be seen at the centers of the nodules. Thereafter the histological picture remained fairly constant for a few days. On the ninth day, the nodular organization of the cortex had begun to lose definition. The nodules were increasingly indefinite on succeeding days, and the size of the node diminished.

*Experiments Involving Different Serological Types of the Virus*

Another series of experiments was done to confirm the specificity of the reaction. Each rabbit received 0.2 ml of a PR8 vaccine in the right foot-pad, and 0.2 ml of a Lee vaccine in the left foot-pad. These strains are respectively of

type A and type B influenzal virus, and do not cross-react serologically. After suitable intervals of time the lymph, lymphnode, and serum were collected and all specimens were tested for their antibody-content to both PR8 and Lee viruses. In testing the specimens obtained it was found that the pattern of appearance of

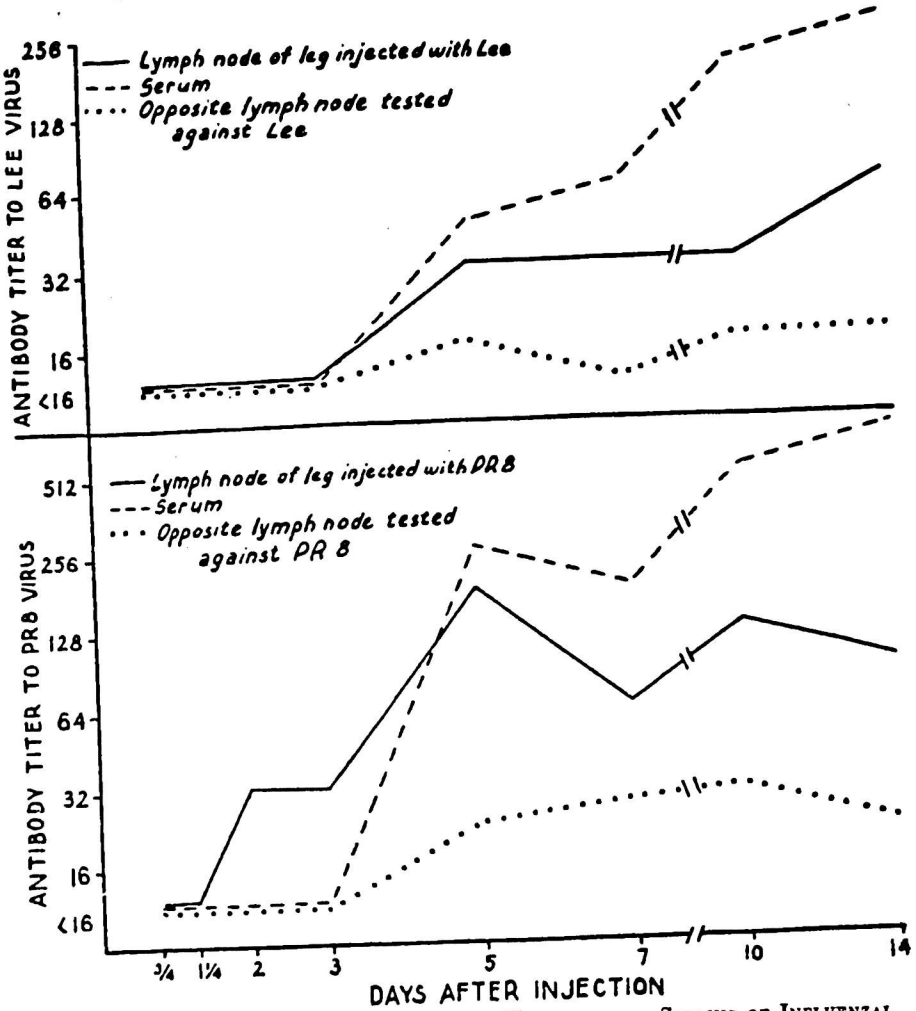


FIG. 2. ANTIBODY-TITERS TO HOMOLOGOUS AND HETEROLOGOUS STRAINS OF INFLUENZAL VIRUS IN BOTH POPLITEAL LYMPHNODES OF RABBITS FOLLOWING THE INJECTION OF SEROLOGICALLY DISTINCT STRAINS OF THE VIRUS INTO THE RESPECTIVE FEET OF EACH RABBIT: COMPARISON WITH SERUM-TITERS

antibodies to the virus injected was similar to that described above. In the early days after injection (2 to 4 days) antibodies were found in the right lymphnode against PR8 exclusively, and in the left node only against Lee. The antibody-titer of the serum lagged behind that of the lymphnode as had been the previous

experience. As the titer of antibody in the serum against PR8 and Lee neared its peak it was found that the lymphnode of the right leg, which had been injected with PR8, contained antibodies to Lee virus in low titer. Similarly, the lymphnode of the left leg, which had been injected with Lee virus, began to show anti-

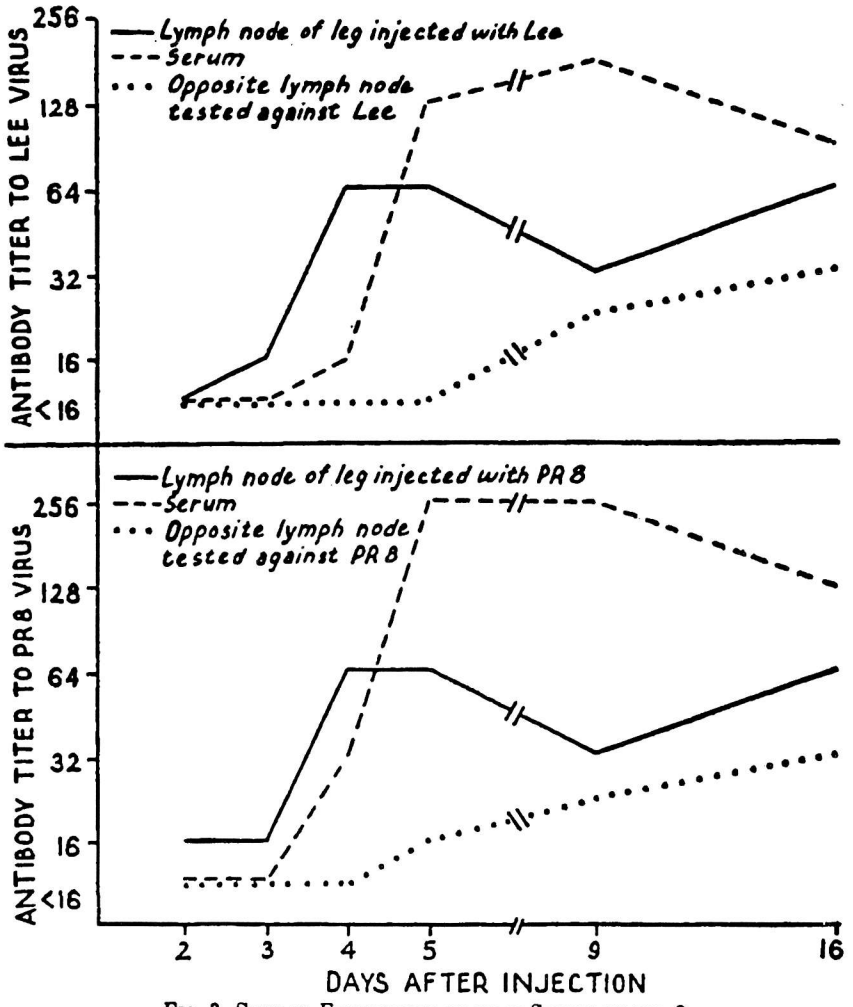


FIG. 3. SIMILAR EXPERIMENT TO THAT SHOWN IN FIG. 2
Although these results indicate that the PR8 preparation was of less antigenic potency than the preparation used in the previous experiment, the time-relations in rise of antibody-titers are seen to follow the same pattern.

bodies to PR8 virus. The level of antibody found in the respective lymphnode-extracts against the heterologous virus generally was about 10 to 15 per cent of the level of serum-antibody. This percentage agrees well with the percentage of serum-antibody content to be found in various tissues of the body found by

Freund (17). Since each leg had been injected with an antigen and each popliteal lymphnode was the site of physiological activity above the normal level it might be expected that the antibody circulating in the serum would be fixed to a greater extent, perhaps, in the active lymphnode than in another tissue not directly involved.

Figs. 2 and 3 illustrate two of the experiments described above. The development of antibodies to the homologous and heterologous viruses are traced for the lymphnodes and the serum. As can be seen, the general pattern is similar to that of earlier experiments. A difference in the properties of the preparations of antigen may be observed in that the rise of titers in all the tissues examined is earlier in one experiment than in the other. Within each experiment, however, antibody is seen to have appeared in the local lymphatic system before it was observed in the serum.

TABLE II

*Range of Individual Variations in Titer of the Specimens Tested in the Early Days After Injection of Antigen*

| RABBIT NO. | DAY OF COLLECTION | TITER OF | | |
|---|---|---|---|---|
| | | Lymph | Lymphnode-extract | Serum |
| 328 | 2 | 128 | 32 | <16 |
| 317 | 4 | 384 | 96 | 64 |
| 341 | 3 | 256 | 128 | 16 |
| 330 | 4 | 384 | 256 | 16 |
| 316 | 3 | 64 | 48 | <16 |
| 340 | 2 | 64 | 64 | 16 |
| 214 | 3 | 16 | 32 | <16 |

*The Concentration of Antibody in Lymph-cells and Lymph-plasma*

For a finer analysis of the source of the antibodies found, the lymph collected from the efferent lymphatic vessel of the popliteal lymphnode was separated by centrifugation into lymph-cells and lymph-plasma, and each fraction was tested for its content of antibody. As was noted above, the pattern-form of neutralization test against viral hemagglutinins was chosen here because of the small volumes to which this test could be adapted. Since a direct comparison between each lymph-cell sediment and its own lymph-plasma was the purpose of the experiment, the plasma of each specimen was tested in parallel with the cells, in an exactly similar micro-test. Since it was also of importance to compare the titers of lymphocytic extracts with titers found generally in this study, parallel tests of the lymph were done involving the conventional volumes of reagents. Table I shows the data obtained in this experiment.

It is seen that the titer of antiviral antibodies in the contents of the lymphocytes is in all cases higher than that in the plasma of the same specimen, and that this difference is greatest in the earlier days. The table also gives the corresponding total cell-counts of the lymph, which are fairly representative of the

counts observed throughout this study. It is also seen that the reaction of the local lymphatic system, both in terms of concentration of antibody and of cell-count in efferent lymph, is not categorically different in animals which received 2-fold concentrated allantoic fluid from that in animals injected with ten times that amount of antigen.

## DISCUSSION

The data presented show that following the injection of inactivated influenzal virus into the foot-pad of rabbits there is a general burst of activity of the local lymphatic system, characterized by a marked enlargement of the sole draining lymphnode of the area and an increase in the total number of lymphocytes in the efferent lymph from that node. The enlargement of the node is due to lymphocytic hyperplasia which is at first diffuse and then becomes organized into the characteristic follicular structure. At the same time antibodies to the viral protein injected appear in the substance of the lymphnode and in the lymph emerging from that node. The antibodies in these tissues are frequently found earlier and, in early days, in higher concentration than in the blood-serum. No antibodies to influenzal virus were found under the condition of these serological tests in lymphnodes of legs opposite to the leg injected, lymphnodes of unmanipulated rabbits, lymphnodes derived from rabbits which had received antigens other than influenzal virus and sera taken prior to injection with influenzal virus. The set of observations extends findings made previously in the same system with bacterial and other cellular agents (4–7). The use of influenzal virus inactivated beyond the range of infectivity eliminates the question of multiplication of the agent and provides data for a representative of another group of proteins, those of viral agents.

A number of the observations made in this study agree with those made earlier by McMaster and Kidd who used active vaccine-virus and employed another system of lymphatic tissue, also in the rabbit. Similarly Burnet and Lush were able to demonstrate neutralizing antibodies in mediastinal lymphnodes of mice infected with influenzal virus.

The antibody-titers reported here have primarily a relative significance, since their measurement is used to point to the primary site or source of the antibodies found. The actual titers could have been varied at will, by altering the number of units of virus against which the neutralization-tests were performed. The amount of virus was fixed, as was the lower limit of dilutions of specimens employed in the tests, so as to preclude on a quantitative basis any non-specific reaction. In the presence of 8 units of virus a 1:16 dilution of serum, lymph or lymphnode-extract was found to be above the limits of non-specific interference by normal tissues or those stimulated by other antigens, so that in the tests as reported each titer can be accepted without reservation as a specific-antibody titer.

Further evidence of specificity was afforded by the experiments in which opposite legs of each rabbit received injections of different serological types of influenzal virus. The differences in titers to the homologous and heterologous virus are clearly marked and in relation to the existing titer in the serum to that

antigen, the concentration of heterologous antibody is quite in accordance with what would be expected as a result of Freund's investigations on the distribution of serum-antibodies in the tissues. In fact it may be noted, in following the homologous-antibody titers of extracts of a given lymphnode through successive days, that the mean titers begin to decline toward the end of the first week, only to increase again in a measure thereafter. It may well be that the later rise represents a summation of the declining rate of antibody-production within the node itself plus an increasing rate of concentration of antibody from the serum. The demonstration of antibodies in higher titer in the local lymphatic system than in the serum, in the early days of antibody-production, is not a necessary condition for the demonstration of antibody-production by the lymphatic tissue for two reasons. First, the concentration of a substance at a given time need not be higher at a site of production than in a reservoir into which it is being drained. Second, unless the amount of antigen injected is quite small there is very probably antibody-formation in lymphnodes proximal to the popliteal as a result of antigen-specific soluble material passing through the popliteal node (9). Under these circumstances the finding of antibodies earlier and in higher concentration in the local lymphatic system than in the serum is particularly significant. It should be noted that in the case of the experiment summarized in fig. 1 both legs were injected with the same antigen, so that the serum was receiving antibody simultaneously from two sources of supply. The greater antibody-titer in lymph and lymphnode-extract than in the serum in the early days of this experiment has, then, even a greater significance as to the lymphatic source of the antibodies found.

The concluding proof of the formation of antibody to viral protein in the lymphatic system is the evidence for the lymphocyte itself as a primary source of the antibody. The titers in contents of lymphocytes was found to be as high as 8192, and even this observed value is probably not as high as the true titer, since the volumes on which calculations of volume were based were derived from a graph which agreed closely with one based on hematocrit-readings of packed cells. Inasmuch as packed cells contain interstitial fluid caught among them, the true volume of lymphocytes is certainly lower than the packed-cell volume, and the true titer of lymphocyte-contents is correspondingly higher. Even the values recorded, however, show a ratio of as much as 16 to the titer of lymph-plasma of the same specimens. It is considered of additional significance that this ratio is greatest at the time of the greatest rate of increase of antibody in the lymphatic system, for it would be logical to expect, at that time, the greatest ratio between the concentration of antibody in its primary source and that in its secondary site. No repetition was undertaken here of the demonstration that the antibodies in the lymphocytes were not, in all probability, concentrated in some way by those cells from the lymph-plasma, since this rather laborious demonstration had comprised the major portion of a previous communication (7).

## SUMMARY

Following the injection of inactivated influenzal virus into the foot-pad of the rabbit antibodies to the viral protein can be found in the popliteal lymphnode,

which drains the site of injection, and in lymph obtained from the efferent lymphatic vessel of that node. These antibodies are found earlier in the local lymphatic system that in the serum and in higher titer until the 4th day after the injection. Thereafter the serum-titer rises above that in the lymphatic tissues.

At the same time there is a burst of activity in the local lymphatic tissue. There is marked enlargement of the lymphnode, almost entirely of cortical tissue. This consists of diffuse lymphocytic hyperplasia which very soon becomes organized into the conventional follicular structure of lymphnodes. The absolute and relative count of lymphocytes in the lymph emerging from the popliteal lymphnode rises sharply.

Injection of serologically distinct strains of influenzal virus into the two legs of individual rabbits give results which corroborate the specificity of the antibodies produced.

On separation of lymph emerging from the popliteal lymphnode into cells and plasma and testing each for antibody-content, it is found that the titer of antibody in the lymph-cells exceeds that in the lymph-plasma, by ratios as high as 16:1. This ratio is found to be highest at the time when the rate of increase of antibody-content of the local lymphatic system is greatest.

These findings are interpreted to mean that the lymphocyte can be a primary source, or site of final synthesis, of antibodies to viral protein. This conclusion is in keeping with those of earlier studies in which a similar role was demonstrated for the lymphocyte in the formation of antibodies to bacterial and cellular antigens.

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