FORMATION OF TETANUS ANTITOXIN BY SPLEEN AND LYMPH NODE INTRAOCULAR TRANSPLANTS**

Evidence for antibody formation by various cells, tissues, and organs has come mainly from studies employing the following methods: (i) extracts of tissue cultures,1,2,3 (ii) cannulization of afferent and efferent lymph,4,5 (iii) extracts of cells, tissues, and organs,6,7,8 (iv) extirpation of the spleen,9,10 and (v) roentgen-radiation of animals with lead-protected mobilized spleens.11 Although the reports arising from these are controversial, there now seems but little doubt that spleen and lymph nodes actively producing antibody in the intact animal will continue to do so for a few hours following removal and transfer to tissue culture. There is also fairly uniform agreement that the addition of antigen to a tissue culture does not increase, but may prevent, the formation of antibody.

The present study indicates that the anterior chamber of the eye of the irradiated mouse provides a good site for studying antibody formation by isolated tissue transplants. A distinct advantage is that the intraocular transplant is nourished in a more physiological manner than is possible with tissue culture methods. Tetanus antitoxin production by the transplants was studied because very small amounts of this antibody can be detected with good quantitative accuracy. The anamnestic response was employed to test the capacity of various intraocular transplants to produce this antitoxin. The advantage of employing this recall response lies in the greatly increased amount of antitoxin produced by a given quantity of antigen and its much more rapid production.

MATERIALS AND METHODS

Spleen and lymph node tissue from the donor was removed 30 to 60 days after the primary or secondary antigenic stimulus, as described in the various experiments. The tissue fragments, about 1 mm. in diameter, were transplanted into the anterior chambers of mice within one to three hours after irradiation. All recipient mice and irradiated...
ated controls in the experiments reported here received 600 Rep. whole-body cobalt-60 irradiation. This amount of irradiation normally causes no deaths in our four-week-old mice. The LD₅₀ for mice of this age is 750 Rep. on our source. Irradiation of the recipient mice served the two-fold purpose of increasing the percentage “take” of the transplants and of practically abolishing antibody production by the recipient animals, thus increasing the probability of detecting the amount of antibody produced by the transplanted tissue. Six to 12 days after the transplants were established, the mice were injected intravenously with 0.2 ml. of a 1:4 dilution of fluid toxoid and 9 to 11 days later, they were bled. At this time, the mice with regressing transplants were discarded. The tetanus toxin* used in these experiments was ammonium sulphate precipitated and dried. A 2% solution of toxin was made in 50% buffered (pH 7.4) reagent glycerine and stored in ampoules at —18° C. If the precaution is taken always to use a dry syringe for removal, the toxin will remain stable for many months under these conditions and the original titration can be relied upon. Titration of the toxin was done in three stages: rough, semi-fine, and fine. Ten mice of our inbred strain were used for each dilution in the final titration. The antitoxin combining power was determined by comparing it to a standard obtained from the Institute of Health. The titration of the antitoxic serum from the various experiments was always done in at least two stages, a rough and a fine, and sometimes in three stages. This greatly decreases the number of mice required. We found the inbred strain of mice to be very uniform in their reactions to tetanus toxin and that two to four mice for each mixture gave very uniform results. Except for the very low unitage, the serum was varied and the toxin was held constant. For the very small amounts of antitoxin, it was more practical to hold the serum constant and vary the amount of toxin. All dilutions of toxin and serum were made in beef heart infusion broth. The titrations were read at the end of four days and we found it more accurate to use minimal paralysis instead of death as an end-point. However, the lethal end-point could be used without making a significant difference in the results.

**EXPERIMENTAL**

The first experiment was designed to determine whether tetanus antitoxin formation by lymph node and spleen intraocular transplants from immune donors into irradiated recipients could be detected. The donor mice had received one injection of 0.05 ml. alum-precipitated tetanus toxoid 30 days prior to the removal of the tissue for transplantation. These animals had from 0.125 to 0.250 International units of tetanus antitoxin at the time the tissues were taken for transplantation. The experiment was designed to test the formation of antitoxin by the transplant without further antigenic stimulation, as well as the effect of a secondary stimulus consisting of 0.2 ml. of a 1:4 dilution of fluid tetanus toxoid injected intravenously into the tail vein of the recipient irradiated mice 10 days following transplantation.

* We are indebted to Lederle Laboratories, Pearl River, New York, for a generous supply of this toxin.
From Table 1, it is clear that lymph node tissue which was given a secondary stimulus responded by producing tetanus antitoxin, whereas lymph node transplants not so stimulated did not produce detectable quantities. The spleen tissue possibly produced a very small amount after stimulation, and none without stimulation. Frontal sections through the anterior chamber of the eye, containing lymph node and spleen transplants, are shown in Figure 1 and Figure 2 respectively. These sections were taken seven weeks from the time of transplantation.

### Table 1: Tetanus Antitoxin Production by Lymph Node and Spleen Transplants in Anterior Chambers of Eyes of Irradiated Mice

Spleen and lymph node donor tissues were taken from mice that had received only one injection of alum-precipitated tetanus toxoid subcutaneously 30 days prior to the transplantation.

| 20 mice in each series received in a.e. of each eye | Toxoid treatment 10 days after receiving transplants to a.e. of eyes | Fraction of Intern. unit ml. of pooled serum from group | No. of MLD of tetanus toxin neutralized by 1 ml. of pooled serum |
|----------------------------------------------------|------------------------------------------------------------------|----------------------------------------------------------|---------------------------------------------------------------|
| I—spleen                                           | Not inj.                                                         | < .00004                                                  | < 1.5                                                         |
| II—spleen                                          | Fluid tetanus toxoid i.v.                                        | .00007                                                   | 2.0                                                           |
| III—lymph node                                    | Not inj.                                                         | < .00004                                                  | < 1.5                                                         |
| IV—lymph node                                     | Fluid tetanus toxoid i.v.                                        | .01                                                      | 400.0                                                         |

Because of the marked destructive action of the gamma irradiation on the lymph nodes and spleen of the recipient mice, the second experiment was designed to confirm the first one and also to test the possibility that spleen transplanted to one eye and lymph node transplanted to the other eye might have a stimulating or synergistic effect on each other. No increase in antitoxin formation resulted from a combination of lymph node and spleen transplants (Table 2). These mice had less antitoxin than those in Group II which had lymph node in the anterior chamber of each eye. It is also clear that the transplant must receive a secondary stimulus in order to produce a detectable amount of tetanus antitoxin. The amount of antitoxin produced by the mice in Group II is only 0.1 as much as in a similar group, Group IV in Table 1. The cause of this variation is not yet determined.

The very doubtful formation of tetanus antitoxin by the spleen transplants was somewhat surprising in view of the very definite production by
Fig. 1. Frontal section of lymph node transplant in anterior chamber of the eye seven weeks after transplantation. Stained with hematoxylin and eosin.
FIG. 2. Frontal section of spleen transplant in anterior chamber of the eye seven weeks after transplantation. Stained with hematoxylin and eosin.
the lymph node transplants. It should be emphasized here that in the first experiments the donor mice received only one subcutaneous immunizing injection of alum-precipitated tetanus toxoid. Under this condition, lymph node tissue definitely produced antitoxin, whereas antibody formation by splenic tissue was doubtful. The possibility that spleen from donors having had both a primary and secondary stimulus might be better for transplants of this tissue was tested in the third experiment. The donor mice were given

| Table 2: Tetanus Antitoxin Production Following Combined Lymph Node and Spleen Transplants from Immune Mice to Irradiated Mice |
|---|---|---|---|
| No. of mice receiving transplants | Toxoid treatment 9 days after receiving transplants to a.c. of eyes | Fraction of Intern. unit/ml. of pooled serum from group | No. of MLD of tetanus toxin neutralized by 1 ml. of pooled serum |
| I—20, no transplant | Fluid tetanus toxoid i.v. | < .00004 | < 1.5 |
| II—25, lymph node* | " " | .001 | 40.0 |
| III—25, spleen* | " " | < .00004 | < 1.5 |
| IV—25, lymph node in a.c. of one eye and spleen in a.c. of other eye | " " | .0003 | 12.0 |
| V—25, lymph node* | Not inj. | < .00004 | < 1.5 |
| * In a.c. of each eye. |

0.05 ml. alum-precipitated tetanus toxoid subcutaneously for the primary stimulus and 0.05 ml. of fluid toxoid intra-abdominally 22 days later for the secondary stimulus. The splenic tissue was removed for transplantation six days following the second injection. The donor mice having received two such injections had from 7.5 to 20 International units of tetanus antitoxin per ml. of serum 10 days after the second injection. Because of the antitoxin content of the donors' tissue, it was desirable to have a control on the amount of antitoxin carried over by the tissue transplants. For this purpose, twice-frozen and thawed splenic tissues from the immune donors were transplanted to the mice in Group III, Table 3. The recipient animals in Groups I and III were given 0.05 ml. fluid tetanus toxoid intravenously 12 days after receiving the transplants. Those in Group II were not injected
with toxoid, serving as controls for the amount of antitoxin formed by the live spleen transplants over a period of 12 days without further antigenic stimulus. Under the conditions of this experiment there is no doubt that the spleen transplants from twice-stimulated donors can form antitoxin in the irradiated recipients without any further antigenic stimulus if the donor tissue is taken during the active phase of antitoxin production. However, the intravenous injection of toxoid into the recipients more than doubled the amount of antitoxin produced. The antitoxin content of the serum from the

| Table 3. Production of Antitoxin by Spleen Transplants from Donors Receiving Alum-Precipitated Toxoid Subcutaneously for 1st Injection and Fluid Toxoid Intra-abdominally for the 2d Stimulus 22 Days Later. |
| --- |
| Twice-frozen and thawed spleen from similar donors was used as control. Donor tissue was taken 6 days after 2d injection. |

| No. of mice receiving toxoid treatment 12 days after receiving transplants to a.c. of each eye | Fraction of Intern. unit/ml. of pooled serum from group | No. of MLD of tetanus toxin neutralized by 1 ml. of pooled serum |
| --- | --- | --- |
| I—25, fresh spleen transplants | Fluid tetanus toxoid i.v. | .00125 | 50.0 |
| II—25, fresh spleen transplants | Not inj. | .00047 | 19.0 |
| III—25, frozen and thawed spleen transplants | Fluid tetanus toxoid i.v. | .00004 | 1.5 |

mice in Group III indicates that only a very small amount of antitoxin is passively introduced by the frozen and thawed transplant.

Whole-body irradiation of the donor mice will greatly reduce the percentage and duration of “takes” of spleen transplants in irradiated recipient mice. Therefore, to control better this carry-over of antitoxin by donor tissue, an experiment was designed to use spleens from twice-stimulated, irradiated donors. The irradiated donors were given 600 Rep. cobalt-60 whole-body irradiation one to three hours before the tissues were removed for transplantation. Spleens were taken from the immune donors 12 days after the secondary stimulus. The results of this experiment are represented in Table 4.

As can be seen, the serum from the mice in Group I and Group II—the recipients of the immune spleen from irradiated donors—had a just detectable amount of antitoxin. The injection of toxoid intravenously did not
increase the amount of antitoxin. The animals in Group I have no more antitoxin than those in Group II. In contrast to this, the mice in Group III with spleen transplants from non-irradiated immune donors and which had been given toxoid intravenously seven days after receiving the transplant had 0.001 International units of antitoxin per ml. of serum. The antitoxin content of the serum from the animals in Group IV is greater than in the control Groups I and II. Although this difference is not great, it exceeds the experimental error in titration, indicating that the spleen from non-

| Table 4. Comparison of Antitoxin-forming Ability of Spleen Transplants from Irradiated, Antigenically Twice-stimulated Donors to Spleen Transplants from Similarly Immunized, but Non-Irradiated Donors |
|-----------------------------------------------|-----------------|----------------|
| No. of mice receiving spleen in each eye      | Toxoid treatment| Fraction of    |
|                                              | 7 days after    | Intern. unit/  |
|                                              | receiving       | ml. of pooled  |
|                                              | transplants     | serum from group|
|                                              | to a.c. of eyes |                |
| I—19, from irradiated donors                 | Fluid tetanus   | .00004         |
|                                              | toxoid i.v.     | 1.5            |
| II—18, from irradiated donors                | Not inj.        | .00004         |
|                                              |                 | 1.5            |
| III—21, from non-irradiated donors            | Fluid tetanus   | .001           |
|                                              | toxoid i.v.     | 50.0           |
| IV—21, from non-irradiated donors             | Not inj.        | .0001          |
|                                              |                 | 4.5            |

irradiated donors, without further stimulation, and after having been transplanted to an irradiated host, continued to produce a small amount of antitoxin.

It may be well to point out that the splenic tissues were removed from the immune donors in this experiment 12 days after the secondary stimulus, whereas they were removed six days after such treatment in the experiment illustrated in Table 3. The more active antitoxin formation on the sixth day as compared to the twelfth day may explain the difference between the comparable Group II, Table 3, and Group IV, Table 4.

When donor mice immunized by a single subcutaneous injection of alum-precipitated tetanus toxoid were used in the first two experiments reported here, there was a clear-cut difference between the production by the lymph
node and spleen intraocular transplants. The results with lymph node were conclusive. The formation of antitoxin by splenic tissue under these conditions was doubtful. However, when this was used from donors that had received 2 immunizing injections, there was no doubt that these transplants could form antibody after being transplanted into the eyes of irradiated mice. Therefore, it was desirable to compare twice-stimulated lymph

and spleen tissue for their ability to produce antitoxin. The results of this experiment are given in Table 5. As can be seen, the amount of antitoxin produced by both the lymph node and spleen transplants is very much greater than in any of the previous experiments. This was true where the transplant was not stimulated as well as where an intravenous injection of fluid toxoid was given to the recipients 6 days after receiving the transplants. It is quite evident from an examination of this table that an antigenic stimulus greatly increases the formation of antitoxin by both transplants. One might conclude from this experiment that spleen was a much better antitoxin-forming organ than lymph node. We do not believe that such a conclusion should be made in view of the variable results obtained in the

| No. of mice receiving in a.c. of each eye | Toxoid treatment 6 days after receiving transplants to a.c. of eyes | Fraction of Intern. unit/ml. of pooled serum from group | No. of MLD of tetanus toxin neutralized by 1 ml. of pooled serum |
|----------------------------------------|---------------------------------------------------------------|----------------------------------------------------------|---------------------------------------------------------------|
| I—24, lymph node                        | Fluid tetanus toxoid i.v.                                    | .004                                                     | 150.0                                                         |
| II—24, lymph node                       | Not inj.                                                     | .0009                                                    | 37.5                                                          |
| III—24, spleen                          | Fluid tetanus toxoid i.v.                                    | .02                                                      | 830.0                                                         |
| IV—24, spleen                           | Not inj.                                                     | .0025                                                    | 100.0                                                         |
| V—20, no transplant—Irradiated controls | Fluid tetanus toxoid i.v.                                    | < .00004                                                 | < 1.5                                                          |
| VI—25, frozen and thawed spleen         | "                                                           | .00004                                                   | 1.5                                                           |
different experiments. At present we cannot be sure what the reasons for this quantitative variation may be, but work in progress may at least partially answer it. Whether or not the lymph nodes used for transplanting are regional to the site of the first alum-precipitated toxoid injection may be one of the reasons. Unfortunately, no attention was given to this point in any of these experiments. We believe that the difference between the red pulp and the white pulp of the spleen may also account for the varying amounts of antitoxin produced by the splenic transplants. The recent work of Thorbecke and Keuning would support both of these views.

One should not conclude from these experiments that the spleen is more difficult to sensitize than the lymph node, because the first sensitizing injections were always subcutaneous in these experiments. We are now trying intravenous, as well as intraperitoneal and subcutaneous, sensitization of the donors, in the latter case comparing the lymph nodes regional to the site with those remote from the site.

Two experiments have been done with intraocular transplants of thymus and Peyer's patch tissue from immune mice into irradiated recipients. One experiment was positive for both tissues and one was negative for both.

One experiment has been done with skin transplants. This tissue grows better in the anterior chamber than any other we have tried, but no detectable antitoxin was produced.

The M.L.D. of tetanus toxin and botulinus toxin is the same on a milligram for milligram basis. Because the molecular weight of tetanus toxin is 72,000 and that of botulinus toxin is given as 900,000, we believe that a much smaller amount of botulinus antitoxin can be detected than can tetanus antitoxin. We plan to compare the sensitivity of these 2 antigens for this purpose.

SUMMARY

1. Lymph node and spleen tissue from immunized donor mice will produce tetanus antitoxin when transplanted to the anterior chamber of the eye of gamma irradiated recipients.

2. More antitoxin is formed by the transplants from immunized donors when the recipients are injected intravenously with fluid tetanus toxoid.

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