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Delayed Antibody Synthesis in Mice after Transfer of Immune Peritoneal Fluid Cells

E. WEILER

The Institute for Cancer Research, Philadelphia 11, *Pennsylvania, U.S.A.*

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Summary. Ascites fluid, rich in bacteriophage-neutralizing antibody, was produced when mice were treated first, with lethal or near-lethal whole body X-radiation; secondly, intravenous injection of spleen cells from donor mice immunized against bacteriophage; and thirdly, with an intraperitoneal injection of bacteriophage in Freund's adjuvant. The 'immune ascites cells' were washed and transferred to other mice without further addition of antigen. The production of phage-neutralizing antibody in recipient mice showed the following properties.

(1) The highest rate of antibody synthesis occurred between the 5th and the 11th day after cell transfer. In contrast, spleen cells similarly transferred gave rise to antibody formation with the maximum rate of synthesis immediately after transfer.

(2) The antibody formation occurred essentially only in isologous recipients, not in homologous ones, whether the latter were pre-immunized against cells of the donor strain or not. With spleen cells, antibody synthesis was not impaired in homologous hosts for about 4 days after transfer, if the hosts were not pre-immunized against the donor strain.

(3) Freezing and thawing of the donor cells prior to injection into the hosts abolished subsequent antibody synthesis.

(4) Irradiation of the cells with 650 R. abolished antibody formation after transfer.

(5) Whole-body irradiation of the recipient mice resulted in increased antibody formation.

(6) When immune ascites cells were injected into newborn mice, high levels of antibody were found 13 days afterwards.

It is concluded (a) that the population of immune ascites cells carries both the specific information and the stimulus for antibody synthesis, and (b) that the antibody-forming apparatus is not yet present in a functional state at the time of transfer, but develops several days afterwards in the host mice.

INTRODUCTION

In mice, ascitic fluid with a high content of antibody can be induced by intraperitoneal injections of antigen in Freund's adjuvant (Anacker and Munoz, 1961). In the present paper, a modification of the original method of Munoz is introduced which consists essentially of lethal whole-body X-irradiation of the animals, followed by the grafting of spleen cells prepared from previously immunized mice, and the subsequent application of the antigen in Freund's adjuvant.

This study is concerned with the question of whether or not the cells of ascitic fluid (monocytes, polymorphonuclear leucocytes and lymphocytes) play any role in the production of antibody. They were therefore washed and transferred to recipient mice without any further contact with antigen. Antibody production was observed in the recipients; this production displayed a different time course from that following transfer of spleen cells.

To decide whether the capacity to produce antibody after transfer is a property of the transferred cells alone, or whether free antigen plays any role, experiments were designed to answer the following questions:

(1) Do the cells have to be viable, and for how long; (2) do the recipient animals have to be immunologically competent themselves? Freeze-thawing or irradiating the transferred cells; comparison of the results in genetically homologous (allogeneic) versus isologous (isogeneic) cell-recipient combinations; and the use of irradiated or newborn hosts, were the methods employed.

MATERIALS AND METHODS

Antigens

Bacteriophages of the strains T6r$^+$ and T7 were used as antigens. They were obtained from Dr. T. F. Anderson. Their host was *Escherichia coli* B. The general procedures in handling phage were those described by Adams (1959). The high titre phage stocks used for immunization were prepared by the agar overlay method, purified by differential centrifugation, freed from bacterial debris by antisera against *E. coli* prepared in mice, and stored in the phosphate-buffer described by Vielmetter and Wieder (1959). Crude extracts from confluently lysed agar overlay plates were used for the phage neutralization assays.

Phage Neutralizing Assay

Serum samples were withdrawn at intervals from the orbital venous sinus of mice by the technique of Stone (1954). The assay to determine their phage neutralizing activity was taken from Adams (1959) but differed in that an aliquot of the reaction mixture was not passed through a 100-fold dilution step before adding a sample to the soft agar tube. Instead, the reaction mixtures containing the serum dilution and a phage dose of 200 plaque-forming units were contained in teflon depression plates and had a total volume of only 0·06 ml. After the incubation period they were washed as a whole into tubes containing 2·5 ml. of soft agar, and plated. This is a 30-fold dilution step, and therefore any additional neutralization of phage after plating and before phage adsorption onto bacteria would be negligibly small, especially since a relatively long time was allowed for the reaction to proceed in the reaction mixtures before plating. This reaction time was 60 minutes in a 37° waterbath. Additional time spent in pipetting at room temperature (the same for all the samples in an experiment) was multiplied by 0·65, the empirically determined correction factor, and added to the reaction time for computing the neutralization constant K.

The neutralizing activity of sera was expressed as $K = 2{\cdot}3D{\cdot}t^{-1}{\cdot}\log P_0/P_t$ min.$^{-1}$ (D, serum dilution factor; t, corrected reaction time; P_0, plaque counts in controls without antiserum, P_t, plaque counts after incubation with antiserum) (Adams, 1959). In neutralization tests with serially diluted immune ascitic fluid the log P_0/P_t was found to be

inversely proportional to D, when the degree of neutralization was between 98 per cent and 30 per cent of the phage input, which is the useful range of the assays in the experiments reported here. The lowest serum dilution used was 4, which, at the 30 per cent neutralization level, means a K value of about 0·02. That value was considered as the low limit of quantitative reliability; but lower values were also used for computing averages within groups of mice.

Animals

Female mice, strains C3H-He/NICR and C57BL/6NICR, between 8 and 12 weeks of age, were used in all the experiments.

Production of Immune Ascites Cells

No rigid schedule was followed to immunize the spleen cell donor mice (Stage I in Fig. 1). They were injected intraperitoneally twice with approximately 5×10^{10} plaque-forming units of bacteriophage each time. The interval between the two injections varied from 2 to 8 weeks in different experiments; sometimes the first injection was with Freund's adjuvant. The spleens were excised 5 days after the second injection. They were then first passed through a tissue chopper, which was in principle similar to that described by McIlwain and Buddle (1953). The minced spleens were stirred for 15 minutes in Hanks's solution, or in Hanks's solution containing 0·25 per cent polyvinylpyrrolidone (P.V.P., CalBioChem), and pipetted gently up and down a few times; the tissue chunks were allowed to settle, and the cell suspensions were spun at 1000 rev./min. in an International PR-2 centrifuge for 5 minutes and finally taken up in Hanks's solution with 0·25 per cent P.V.P. White cells were counted in a haemocytometer after treatment with 3 per cent acetic acid and the volume was adjusted so that the desired concentration of cells was obtained.

For irradiation, seventeen mice were placed in a circular plexiglas container, which could be centred under the X-ray machine; individual mouse compartments were at an equal distance from the centre.

The X-ray apparatus, a therapy model by Standard, Chicago, was used with the following characteristics: 200 kV.; 20 mA.; inherent filtration, 1 m. Al; added filtration, none; half-value-layer, 0·5 mm. Cu. The distance from the target to the centres of the mice was 50 cm., and the dose rate at that distance was 37 R./min., as determined by a Victorine dosimeter that was placed in a mouse compartment.

The mice received 750 R. or 850 R. There was no difference with respect to the volume or antibody activity of the ascites fluid produced, or the concentration of cells in it, but after 850 R. more mice appeared to die than after 750 R., and the latter dose was adopted as a routine. Kohn and Kallmann (1956) found 750 R. to kill 90 per cent of C3H mice, and 850 R. 100 per cent.

Each mouse received $38–70 \times 10^6$, but mostly 60×10^6 immune spleen cells intravenously, within 4 hours after irradiation. Shortly afterwards, mice were injected intraperitoneally with about 4×10^{10} plaque-forming units of bacteriophage in phosphate buffer, emulsified with an equal volume of complete Freund's adjuvant (Difco). The injected volume was 0·2 ml.

As a second injection, the same amount of phage in adjuvant was given intraperitoneally. The time interval between the first and the second injection was between 6 and 11 weeks

in different experiments. By that time, some mice had already developed ascitic fluid, which was withdrawn before the second injection was given. Ascitic fluid was withdrawn into a 10 ml. syringe through an 18-gauge needle that had four side holes near the tip. The first ascitic fluid was withdrawn 8–13 days after the second injection.

Preparation of Cells for Transfer

The suspending medium for immune ascites cells was Hanks's P.V.P. solution. This solution is a mixture of 84 per cent Hanks's solution and 16 per cent of a 12·5 per cent solution of polyvinylpyrrolidone (Calbiochem) which had been dialysed against 0·9 per cent NaCl. It was always used, except in some early experiments, when Hanks's solution alone was used, and in the (unsuccessful) experiments described in Section VII of the experimental part, where 10 per cent of anti-T7 immune ascites fluid was added to the Hanks's solution (the cells were anti-T6).

To every 10 ml. of ascitic fluid, 0·1 ml. of heparin (liquaemin-sodium, Organon, 5000 U. per ml.) was added after withdrawal. The cells were sedimented at 1600 rev./min. for 5 minutes, and the pellets were resuspended in 12 ml. of Hanks's P.V.P. The suspensions were layered over an equal volume of a mixture of 2 parts of Hanks's P.V.P. and 1 part of 0·6 M sucrose, and sedimented at 1600 rev./min. for 5 minutes. This washing procedure was repeated twice again. The cells were finally suspended in the desired volume of Hanks's P.V.P. for the injections.

To test the efficiency of the washing procedure, the neutralizing activity of the suspension medium after the last washing was determined in two experiments. The K values were 0·13 and 0·058, respectively. This is three orders of magnitude less than the neutralizing activity of the ascitic fluids. In the experiments with newborn mice (Section V), the resuspended cells were passed through a 90 mesh stainless steel screen fitted into a Swinney syringe filter holder, to remove clumps of mostly fibrous material; then they could easily be passed through a 30-gauge needle.

For X-irradiation, cell suspensions in polystyrene tubes, surrounded by ice, were placed in compartments of the mouse irradiation container (see above). The cells were kept chilled during the preparation procedure up to the time of injection. Occasional checks with toluidine blue (1 drop of 1 per cent solution in 0·8 per cent NaCl, mixed on the microslide with 1 drop of cell suspension) revealed that practically all the cells were intact after the preparation procedure. For the toluidine blue test, the cells had to be removed, by centrifugation, from the Hanks's P.V.P. solution, and suspended in pure Hanks's solution, because Hanks's P.V.P. interferes with the staining of damaged cells.

Absolute and differential cell counts of immune ascites cells were carried out in haemocytometer chambers by a simplified version of the nuclear counting method of Sanford, Earle, Evans, Waltz and Shannon (1951). Cells were sedimented from the ascitic fluid, and the pellets were suspended in a 0·1 M citric acid solution. The suspensions were incubated at 37° for about 1 hour and immediately afterwards (or after storage in the refrigerator) a solution of 0·1 per cent crystal violet in 0·1 M citric acid was added. The volume of added crystal violet solution was half the volume of the cell suspension in citric acid. The stained suspension could be counted directly.

Spleen cells for the experiment in Section 2 were prepared as described under 'Production of immune ascites', but were washed subsequently like immune ascites cells.

Salivary gland cells, used in some experiments for inducing a homograft reaction in recipients of ascites cells, were prepared essentially as described above for spleen cells.

Injection Procedure

Injections were given intravenously. The number of cells for each recipient will be given in the 'Results' section; the injection fluid volume was 0·3 ml. or 0·4 ml. Newborn mice were injected into the sigmoid sinus (Billingham and Brent, 1959) through a 30-gauge needle; the fluid volume in that case was 0·04 ml.

RESULTS

VOLUME AND COMPOSITION OF ASCITES FLUID

In a typical group of seventeen mice (Stage II in Fig. 1), the ascitic fluid, 12 days after the second injection of T6 bacteriophage in adjuvant, averaged 8 ml. per mouse. Its K-value was 76 min.$^{-1}$. The production of ascites continued thereafter for 10 more weeks, during which time the mice were tapped once a week. (It continued after that,

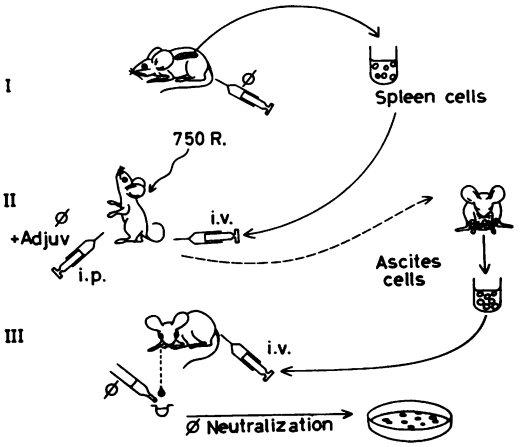

FIG. 1. Basic design of the experiments. Mice of group I are immunized with bacteriophage (Ø) intraperitoneally (i.p.) and intravenously (i.v.). Five days after the second (i.v.) injection spleen cells are prepared. Mice II received (a) 750 or 850 R. whole body X-irradiation, (b) 40×10^6 spleen cells from mice I i.v., and (c) one intravenous injection of phage in Freund's adjuvant shortly after X-ray treatment and a second one several weeks later. A peritoneal exudate develops within 12 days, is withdrawn, and the suspended cells are washed and injected intravenously into mice III. Blood samples taken from the latter at various time intervals after cell transfer are assayed for phage-neutralizing activity.

but the experiment was terminated.) The total fluid volumes over 10 weeks were 50–60 ml. per mouse. K-values were higher in the second and third harvests and then declined slowly.

First harvest ascitic fluid contained $7–14 \times 10^6$ nucleated cells per ml. in different groups of mice. Cells were regenerated between successive harvests, but their concentration became lower in later harvests and reached a plateau value of $1·5–2 \times 10^6$ per ml. from the fifth harvest onwards.

Three classes of cell types could be distinguished:

(1) Monocytes; these usually had indented or strongly constricted nuclei; their cytoplasm was often basophilic, and often vacuolated (macrophages). (2) Polymorphonuclear neutrophils; they were uniform in appearance; their nuclei mostly had five lobules or

more. (3) Lymphocytes; more than half of them were typical small lymphocytes; medium-sized and large lymphocytes occurred less frequently. Very rarely medium-sized lymphocytes with intensely basophilic cytoplasm were seen. The proportion of these cells in first harvests of ascitic fluid from eight groups of mice were as follows: monocytes, 46–63 per cent; polymorphonuclear neutrophils, 19–43 per cent; lymphocytes, 9–23 per cent. These figures are derived from counts made on citric acid fixed cell suspensions. Some of the cells registered as monocytes may have been large lymphocytes.

TRANSFER OF IMMUNE ASCITES CELLS

In these experiments immune ascites cells from C3H donors were washed and injected intravenously into recipient mice (Stage III in Fig. 1). The cells were from the first harvest of ascites, 8–13 days after the second injection of phage in adjuvant emulsion, except in the two experiments mentioned in Section VII. Serum samples were withdrawn at intervals from the recipients, and the level of bacteriophage-neutralizing antibody, expressed as the neutralization constant 'K', was determined each time. In Figs. 2–5, the arithmetical means of the K values from each group of recipients are plotted, together with the ranges of variations between individual values. This was the basic design of all the transfer experiments, with the exception of Experiment II, in which spleen cells from immunized donors were transferred.

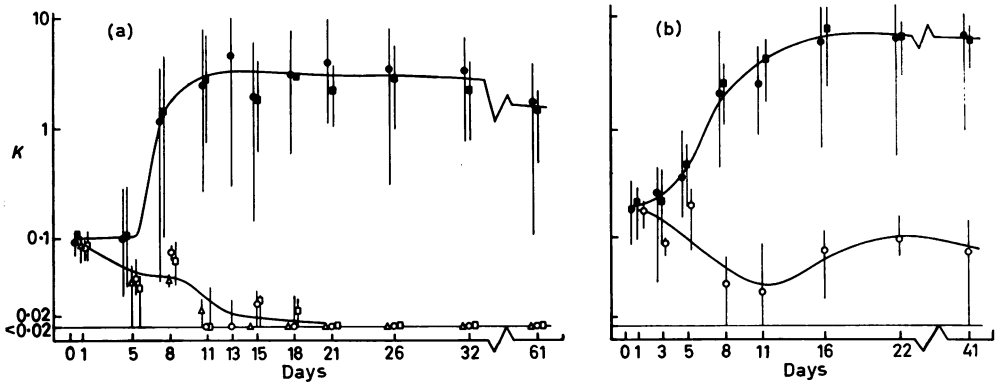

FIG. 2(a) (T6). Experiment I. Antibody production (expressed as phage neutralization constant K) against T6 phage after transfer of 33×10^6 anti-T6 immune ascites cells per recipient mouse on day 0. Four recipient mice per group. Groups 1a (●) and 1b (■): isologous recipients. Groups 2a (○) and 2b (□): homologous recipients. Group 2(△): homologous recipients pre-immunized against the donor strain. Groups 1a and 2a received anti-T7 cells in addition on day 10, see Fig. 2(b) (T7).

(b) (T7). Experiment I. Antibody production against T7 phage following transfer of 22×10^6 anti-T7 immune ascites cells per mouse 10 days after the transfer of anti-T6 cells. Group 1a (●): recipients are the same mice as in group 1a, Fig. 2(a) (T6). Group 1b (■): isologous recipients not pre-inoculated. Group 2 (○): recipients are the same mice as in group 2a, Fig. 2(a) (T6).

Experiments were performed as follows: I and II, recipient mice were of either isologous or homologous strains; IV and V, recipients or cells were treated with X-irradiation; III and V, cells were frozen and thawed before injection into recipients; V, newborn mice were employed as recipients.

Experiment I: Transfer of Immune Ascites Cells to Isologous and Homologous Hosts

The following experiment was designed to reveal: (a) the time course of antibody

formation after cell transfer; (b) the effect of histo-incompatibility between cell donor and host; and (c) any interaction of cells of different immunological specificity, when they are given to the same host. Pertaining to (c), mice were given anti-T6 phage immune ascites cells on day 0, and the same mice received anti-T7 cells on day 10. From day 11 onward, serum samples from these mice were assayed for neutralizing activity against both T6 and T7 phage, and results are represented in Fig. 2(a) (T6) and Fig. 2(b) (T7), respectively.

T6

Groups 1a and 1b. Both groups of isologous C3H recipients received anti-T6 cells on day 0; group 1a received, in addition, anti-T7 cells on day 10. The average K-values with respect to T6 phage remained the same in both groups and are connected by one curve in Fig. 2(b) (T6).

On the first day after transfer of T6 immune ascites cells into recipients, the K-values were 0·1. They remained unchanged until the 5th day, on the average, but differences between individual mice increased during this period. On the 8th day, the average K-value increased more than tenfold, but not all the mice took part in the increased antibody production yet. On the 11th day all mice developed an increased antibody level, and the K-values reached their highest point. Thereafter, they remained on the same level for 3 weeks. Between day 32 and day 62 the K-values decreased, on the average, to about half of their previous maximum levels.

The variation between individual mice within the same groups was large; the highest K-values were up to 40 times as large as the lowest. But each mouse was consistent in giving a high or a low K-value over the duration of the experiment.

Group 2. Homologous C57Bl mice served as recipients for aliquots of the same preparation of immune ascites cells as that used for groups 1a and 1b. The recipients had been immunized against cells of the C3H (donor) strain by the intravenous injection of 40×10^6 C3H-spleen cells.

On the first day after transfer of immune ascites cells the same neutralizing activities were found in the sera of this group, as in groups 1a and 1b. But on subsequent days, the serum activity declined steadily. The curves showed a slight indication of a shoulder on the 8th day, and fell below the level of significance by the 21st day. Presumably, the destructive effect of the homograft reaction on the transferred cells prevented antibody synthesis.

Groups 2a and 2b. Aliquots of the same immune ascites cell suspension were given to (homologous) C57Bl mice which had not been immunized against the C3H (donor) strain. Both groups of recipients received anti-T6 cells on day 0; group 2a received in addition, anti-T7 cells on day 10. Again, no effect on T6-neutralizing activities was found after the superinoculation of anti-T7 cells.

Both groups showed the same initial K-value on day 1 as all the other groups; their serum activities declined from then on in the same fashion as in group 2. The shoulder on day 8 was slightly more pronounced in these than in group 2.

The only agent that could have induced a homograft reaction in these groups, and thus prevented antibody formation, was the transferred immune ascites cells themselves. Even though one would expect the onset of the homograft immunity to be delayed in groups 2a and 2b, as compared to group 2, the reaction of the host against the graft

cells appears to be sufficiently fast to prevent antibody synthesis, with the possible exception of the slight difference on day 8.

T7 (Fig. 2b (T7))

Groups 1*a and* 1*b*. The mice in group 1a are the same as those in group 1a of the T6 part of this experiment: they had received anti-T6 cells on day 0, before receiving anti-T7 cells on day 10. In group 1b, mice had not been pretreated before receiving anti-T7 cells. No difference between the two groups could be observed with respect to antibody formation against T7 phage. One can conclude from this result, that a previous heterologous antibody formation is not responsible for the delay in antibody synthesis after immune ascites cell transfer.

The time course of antibody synthesis against T7 phage was essentially the same as that against T6, but the increase in neutralizing activity of the sera occurred somewhat earlier, and the maximum K-values were higher (Fig. 2b (T7)).

Group 2. This group of (homologous) C57Bl recipients had been immunized against the C3H (donor) strains, in this case, by the injection of anti-T6 immune ascites cells, 10 days before anti-T7 immune ascites cell transfer. As was found with T6 phage, the K-values of the homologous recipients on the first day after cell transfer were the same as those of the isologous recipient groups. And again, they declined from then onwards. After anti-T7 immune ascites cell transfer, however, the homologous recipients retained significant levels of neutralizing antibody over the duration of the experiments; but they were lower, by two orders of magnitude, than those from the isologous recipient groups (Fig. 2b (T7)).

Another experiment was performed, in which anti-T6 immune ascites cells were transferred to (isologous) C3H mice and to (homologous) C57Bl mice. The homologous recipients had been immunized against the C3H strain by the intravenous injection of 3×10^6 C3H salivary gland cells per mouse, 6 days prior to immune ascites cell transfer. The time course of antibody production in the isologous group, and the failure of antibody formation in the homologous recipients, resembled those in the experiment just described.

Experiment II: Transfer of Immune Spleen Cells

Cell suspensions were prepared from the spleen of mice that had first been injected with 10^{11} plaque-forming units of T6 phage intraperitoneally; 11 days later, 5×10^{10} plaque-forming units were again injected. Cell suspensions were prepared 3 days after the second injection. Three groups of isologous recipients (group 1) or homologous recipients (groups 2 and 3), were given 60×10^6 spleen white cells intravenously.

Group 1. C3H immune spleen cells were transferred to C3H recipients (Fig. 3, curve 1). On the first day after transfer, the average K-value was 0·1. On successive days it increased gradually, and reached its highest point at a K-value of 0·22 on day 8. From then on it declined slowly, and was back to a value of 0·1 on day 30, when the experiment was discontinued. The levels of neutralizing antibody attained in this experiment were considerably lower than in the experiments with immune ascites cells.

Group 2. Aliquots of the same spleen cell suspension were injected into fresh (homologous) C57Bl recipients. The resultant phage-neutralizing activities (Fig. 3, curve 2) were essentially the same as those of the isologous recipient group during the first 4 days after cell transfer. Both increased during that time. But while K-values continued to

rise afterwards in the isologous situation, in this homologous recipient group they declined sharply from the 7th day onwards. Curve 2, Fig. 3, fitted to the experimental points as a straight line in its declining portion, yielded a half-life time of approximately 5 days. It may be that the transferred spleen cells are destroyed by a homograft reaction, with cessation of antibody synthesis.

Group 3. The recipients of immune spleen cells in this group were C57Bl mice that had received a quarter of a chopped C3H salivary gland subcutaneously 40 days before immune spleen cell transfer, and 5×10^6 C3H salivary gland cells intravenously 5 days before spleen cell transfer, to evoke a homograft immunity against C3H cells.

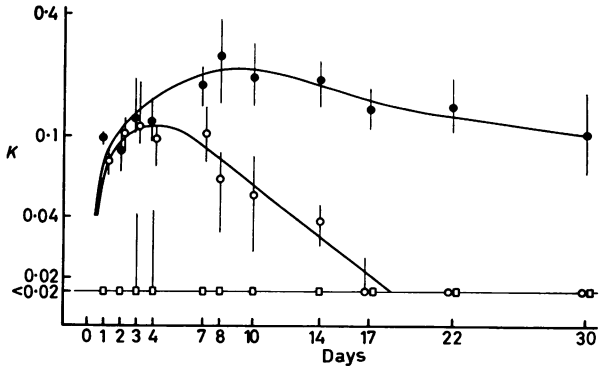

FIG. 3. Experiment II. Antibody production against T6 phage after transfer of 60×10^6 anti-T6 immune spleen cells per mouse on day 0. Group 1 (●): isologous recipients. Group 2 (○) homologous recipients. Group 3 (□): homologous recipients pre-immunized against the donor strain. Four mice per group, except in group 2, where one mouse was lost after day 4 and three remained.

In sharp contrast to the analogous situation in the immune ascites cell transfer experiments, no neutralizing antibody at all could be detected in this experiment, 1 day after immune spleen cell transfer (Fig. 3, curve 3). (One mouse exhibited a small but significant neutralizing activity on days 3 and 4.) The conclusion is that the antibody present on day 1 in groups 1 and 2 was not performed, but was produced after transfer by intact cells which require a compatible environment.

Experiment II shows that the highest rate of antibody synthesis occurs immediately after spleen cell transfer, and not after the 5th day as was the case with immune ascites cells.

Experiment III: The Effect of Freezing and Thawing

Only mice of the C3H strain were used in this experiment. The antigen was T6 bacteriophage.

Group 1. Immune ascites cells from C3H donors were transferred to isologous recipients. The resulting course of antibody formation was essentially the same as in Experiment I, with the highest rate of antibody synthesis occurring between days 4 and 10, when the maximum K-value was reached. There was a slight drop in neutralizing activity between days 1 and 2 (Fig. 4, curve 1).

Three out of the six mice in this group had been immunized intraperitoneally against T7 bacteriophage, in 0·1 ml. of Freund's adjuvant emulsion, 17 days before cell transfer. Since their synthesis of antibody against T6 phage, after anti-T6 immune ascites cell

transfer, was not significantly different from that of the three untreated recipients, the data were pooled for this presentation.

Group 2. The immune ascites cells that were given to this group of recipients were aliquots of the same suspension as used in group 1. But they had been frozen in a solid CO_2-acetone bath, and subsequently thawed in a 37° water bath, three times, shortly before injection into the recipients.

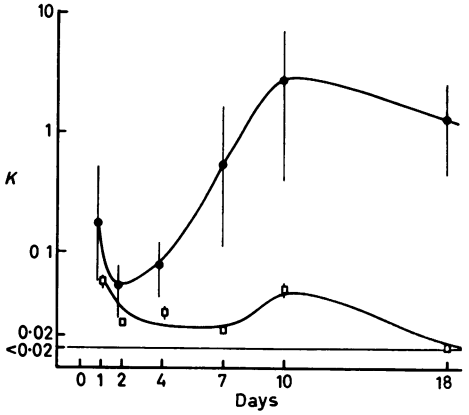

FIG. 4. Experiment III. Antibody production against T6 phage after transfer of 37×10^6 anti-T6 immune ascites cells per mouse on day 0. Isologous recipients. Group 1 (●): transfer of intact cells. Group 2 (□): transfer of frozen and thawed cells. Six mice in group 1, two mice in group 2.

The *K*-values in this group decreased from the first day onwards (Fig. 4, curve 2). Only on day 10 was a slight rise perceptible, which was followed by a drop to the limit of significance by day 18, when the experiment was discontinued. Despite the small number of mice the conclusion seems warranted, that freezing and thawing abolish the capacity of immune ascites cells to cause antibody synthesis to occur after transfer; this conclusion is supported by an analogous experiment with newborn mice (Experiment V).

Experiment IV: The Effect of X-Irradiation on Immune Ascites Cells and on Recipient Mice

Immune ascites cells from anti-T6 donor mice were irradiated *in vitro* with 650 R., 2 hours before they were injected, and the recipients received the same dose as whole-body irradiation, also 2 hours before receiving the cells. Untreated cells and untreated recipients were included in the experiment. 3×10^6 bone marrow cells from isologous, normal female 10-week-old mice were given to both the untreated and the irradiated mice, together with the immune ascites cells, to prevent the premature death of the irradiated animals (Congdon, Uphoff and Lorenz, 1952). All mice in this experiment were of the C3H strain. In order to assess the autochthonous immune competence of irradiated mice in this system, a group of irradiated mice was included, which received the antigen only.

The data are presented in Fig. 5, where the curves are numbered to correspond to the following paragraphs.

Group 1. Untreated cells were given to untreated recipients. The time curve was the same as in Experiment I. The bulk of the antibody was produced between days 6 and 11.

Group 2. Irradiated cells were transferred to untreated recipients. Throughout the experiment, *K*-values were close to the lower limit of significance. No evidence of antibody synthesis was obtained in this group.

Group 3. Irradiated mice received untreated cells. As in group 1, and as in Experiment I, most of the antibody was produced between days 6 and 11. But the levels of antibody were three times as high on day 11 as in the unirradiated recipient group 1, and 5 times as high on day 17. In contrast to all other experiments, no measurable antibody was present on day 3 in the mice of this group.

Group 4. Irradiated cells were transferred to irradiated hosts. As in group 2, no significant antibody formation was observed in this group. This group, and group 2, demonstrate the radiosensitivity of immune ascites cells.

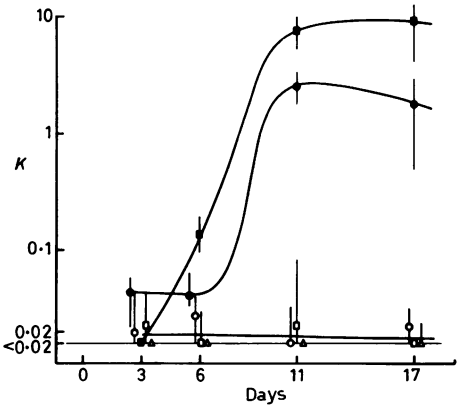

FIG. 5. Experiment IV. Antibody production against T6 phage after transfer of 33×10^6 anti-T6 immune ascites cells per recipient mouse. Isologous recipients. Group 1 (●): untreated cells into untreated recipients. Group 2 (○): irradiated cells (650 R.) into untreated recipients. Group 3 (■): untreated cells into irradiated (650 R.) recipients. Group 4 (□): irradiated cells into irradiated recipients. Group 5 (△): T6 bacteriophage into irradiated recipients. Four mice in each group.

Group 5. About 2×10^{10} plaque-forming units of T6 bacteriophage were injected intravenously into irradiated mice. No antibody was observed. This group of mice show that 650 R. severely depress the primary antibody response of C3H mice toward bacteriophage; and that 3×10^6 bone marrow cells do not significantly compensate for the immunological radiation damage, in accordance with the results of Perkins, Robinson and Makinodan (1961). A control experiment shows that the same dose of bacteriophage, given by the same route, results in a good primary response by the 6th day after injection, when non-irradiated mice are used (Experiment VI, 1).

Experiment V: Newborn Mice as Recipients of Immune Ascites Cells

Five litters of C3H pups received immune ascites cells in 0·04 ml. of Hanks's P.V.P. within 20 hours after birth. They were injected intravenously through the sigmoid sinus, as described by Billingham and Brent (1959). The ascitic cells were harvested from C3H anti-T7 donor mice. In the case of litter *a* the harvest was made 9 days after the second injection into the donor of T7 in Freund's adjuvant; in the case of litters *b*, *c*, *d* and *e*, 10 days after the second injection. All recipients were injected with cells from the same group of donors, and litters *b–e* received injections from the same suspension of washed cells.

Each litter was divided into two groups, one of which was injected with untreated cells; the other served as a control group. The results are presented in Table 1.

On the 13th day after cell transfer, the recipients were killed.

Serum from mice that received untreated immune ascites cells showed high neutralizing activity against T7 bacteriophage, and the average *K*-values were often higher than in adult recipients, although the numbers of injected cells per gramme of recipient mouse (approx. $0{\cdot}7 \times 10^6$) were smaller if the weight at the time of sacrifice (approx. 7 g.) is the basis of computation.

TABLE 1

TRANSFER OF ANTI-T7 IMMUNE ASCITES CELLS TO NEWBORN MICE ($4{\cdot}6 \times 10^6$ CELLS/MOUSE IN LITTER *a*, $5{\cdot}4 \times 10^6$ CELLS IN LITTERS *b–e*)

The age of the recipients at the time of injection is given in the first row; the number of mice is given in each group. Below the average *K*-values, the ranges of individual values are given in parentheses. The data were obtained on the 13th day after birth. The control groups were injected as follows: litter *a*, cells given 850 R. before injection; litters *b* and *c*, 8×10^9 plaque-forming units of T7 phage injected; litters *d* and *e*, cells frozen and thawed before injection.

Litter	*Age (hours)*	*No. of mice*		*K value*	
		Positive group	*Control group*	*Positive group*	*Control group*
a	< 24	5	4	5·2 (2·1–8·2)	0·53 (0·32–0·65)
b	4	5	4	17·4 (8·4–26·9)	0·059 (0·02–0·19)
c	7	4	3	15·7 (1·2–51·0)	< 0·02
d	< 24	4	2	5·1 (0·71–11·0)	0·072 (0·02–0.13)
e	10	6	1	4·3 (0·25–13·1)	< 0·02

Three types of controls were:

(1) The cells were frozen and thawed three times, as in Experiment III, before injection (litters *d* and *e*). Among the three recipients of cells so treated, two had no detectable antibody, and one had the low *K*-value of 0·13. The destructive effect of freezing and thawing on the capacity for antibody formation of immune ascites cells, already reported in Experiment III, was thus confirmed.

(2) The cells were irradiated with 850 R. *in vitro* before injection (litter *a*). The recipient mice had developed low, but significant, neutralizing activities by the 13th day after cell transfer. Their *K*-values lay very close together, with an average of 0·53, i.e. 10 per cent of the positive litter mate group. This differs from the results of Experiment IV, where no antibody was formed after the cells had been treated with the lower dose of 650 R.

(3) The newborn mice were each injected with 8×10^9 plaque-forming particles of T7 bacteriophage in 0·04 ml. of Hanks's P.V.P. (litters *b* and *e*). The mice did not develop significant levels of neutralizing activity, with the exception of one mouse which had the low *K*-value of 0·19.

Experiment VI: Miscellaneous Experiments

1. In Experiment I it was reported that antibody production did not occur when C3H immune ascites cells were transferred to the homologous strain C57Bl, and the failure was interpreted as the result of a destructive effect of the homograft reaction. It was necessary to test whether the C57Bl strain is not inherently inferior to the C3H strain in its capacity to form antibodies against bacteriophage.

One group of each of the two strains, with four female mice per group, were injected intravenously with 2×10^{10} plaque-forming units of T6 bacteriophage, and serum antibody levels were determined 5 days later. The C3H mice had an average K-value of 2·31 (range: 0·8–4·3), and the C57Bl mice had an average K-value of 2·8 (range: 2·2–3·4). The competence for antibody production, therefore, was not inherently different in the two strains of mice used. Besides, as shown in Experiment II, C57Bl mice can support antibody formation by transferred C3H spleen cells during about 4 days after transfer.

2. One experiment was carried out to determine the rate of elimination of passively administered antibody. Four female C3H mice were injected intravenously with 0·3 ml. of ascitic fluid containing T6-neutralizing antibody, diluted with saline to a K-value of 34. Blood samples collected at three different times after injection had the following serum K-values: 7·3 (range: 6·1–9·8) on day 1; 5·5 (range: 4·8–6·1) on day 4; 3·0 (range: 2·5–3·3) on day 11.

These values are compatible with a first order kinetics of antibody elimination, and with an antibody half-life of 8 days.

Experiment VII: Unsuccessful Experiments

In two immune ascites cell-transfer experiments, no significant levels of antibody were observed in the isologous donor–recipient combination. Differences between these two experiments and the successful ones were: (a) the ascites cells were obtained from second or third harvests of the donor mice, instead of from the first harvests, as in the other experiments, (b) only 11×10^6 or 17×10^6 cells, respectively, were transferred in these two experiments. This latter difference, however, should be responsible only for a 70 per cent or 50 per cent lower antibody level, not for complete failure.

DISCUSSION

Mice that had been irradiated, grafted with immune spleen cells, and injected intraperitoneally with bacteriophage in Freund's adjuvant, produce ascitic fluid that contains phage-neutralizing antibody, and a population of monocytes, polymorphonuclear leukocytes, and lymphocytes. When these cells, after washing, are transferred to isologous recipient mice, antibody is produced in the recipients (Fig. 1). During or after transfer the cells were not exposed to antigen, in contrast to most other cell transfer systems (e.g. Dixon, Weigle and Roberts, 1957; Harris, Harris and Farber, 1958; for a recent review on immune cell transfer studies see Cochrane and Dixon, 1962).

The time course of antibody synthesis after transfer displayed unexpected characteristics. From an examination of the data (e.g. Fig. 2a (T6) and 2b (T7)), it is evident that more than 90 per cent of the antibody that can later be found in the recipients' sera begins to appear after day 4. Thereafter, antibody rises sharply, to reach a maximum around days 11 to 16. It then levels off and, after several weeks, begins to decline very slowly.

A small amount of antibody, however, is already present on day 1 in most experiments. It is also found in groups of mice in which the later production of antibody does not occur: in the homologous cell–recipient combination (Experiment I) and in Experiment III, where frozen and thawed cells were transferred. In these cases, the antibody found on day 1 became eliminated during the course of the experiment. The simplest explanation is that day 1 activity represents antibody that was passively carried along when the cells were transferred, and presumably—since the cells were thoroughly washed and the suspending medium contained very low amounts of antibody—within the cells. The

following discussion will be concerned only with the bulk of the antibody which is produced after a delay of several days.

The time curves of delayed antibody activity can be interpreted in two ways: (a) synthesis starts at a very low level soon after transfer, continuously increases in rate, and the resultant activity begins to exceed the level of day-1-antibody on about day 5. (b) There is no synthesis before the 4th day, and antibody production commences then; this would mean that there is a true time-lag phase. A decision between these two interpretations is not yet possible.

The peculiar properties of the immune ascites cell transfer system are illustrated by a comparison with the course of antibody synthesis after transfer of immune spleen cells, 3 days after a secondary antigenic stimulation of the donor mice (Experiment II, Fig. 3). Again, antigen was not added to the system during or after cell transfer. The highest rate of antibody synthesis is found in the first few days after transfer, and the highest level of neutralizing activity is reached by the 8th day. There is no indication of any rate increase after transfer. The antibody that is present on the first day after spleen cell transfer has been synthesized *de novo* in the host animals, as is shown by the finding that no detectable antibody is present on the first day where the transferred cells were subjected to a homograft reaction immediately after transfer (into homologous recipients previously immunized against cells of the donor strain). This spleen cell experiment resembles those performed by Harris and Harris (1954) and by Harris, Harris and Farber (1954). These authors did find a case in which there was a rate increase shortly after lymph-node cell transfer, when they prepared the cells only 1 or 2 days after the injection of antigen into the donor animals. These results can be clearly distinguished from the behaviour of the peritoneal cells in the present experiments: here, the rate increase occurs much later after cell transfer; and the peritoneal cells were harvested from donor mice about 2 weeks after the last antigen injection, at a time when the antibody level in the ascitic fluid of the donors was close to its maximum level. In recent experiments (to be published later), delayed antibody synthesis was observed when the transferred peritoneal cells had been harvested from donor mice as late as 3 months after the last antigen injection.

The difference between the time of antibody synthesis with spleen cells and peritoneal cells is amplified when one compares them in the homologous transfer situation, when the recipients were not pre-immunized against homotransplantation antigens of the donor strain. With spleen cells, antibody reaches a level of the same order of magnitude as in the isologous transfer situation (Experiment II, group 2); with peritoneal cells, antibody is never produced. Splenic tissue contains antibody-producing cells shortly after a secondary antigenic stimulus (Coons, Leduc and Connolly, 1955; Leduc, Coons and Connolly, 1955). After transfer, these cells make antibody early, before homograft immunity in the recipients can develop and destroy the transferred cells. Peritoneal cells, on the other hand, do not produce antibody before about the 4th day after transfer, and by then (after homologous transfer) the recipients have developed homograft immunity and destroy them (Experiment I, groups 2a and 2b).

The time course of antibody synthesis in the peritoneal cell transfer system is not unlike that found in the primary immunization of animals. Since the cell donors have received antigen, the possibility exists that the antibody production in the recipients is due to a primary immunization by free antigen carried along with the transferred cells. This point was clarified as follows.

(1) (a) Antibody synthesis is abolished when the peritoneal cells are transferred to recipients of a homologous strain, which is known to display a strong homograft reaction against grafts from the donor strain (Experiment I). Cells should be destroyed in this situation, but free antigen should not be affected in its immunogenic activity. The effect of the homograft reaction on antibody producing cells has been described by Harris *et al.* (1958) and by Doria, Goodman, Wright, Gengozian and Congdon (1962).

(b) Freezing and thawing the cells during transfer prevents later antibody synthesis (Experiments III and V). This treatment, again, should kill the cells, but not affect the immunogenic properties of bacteriophage.

(c) 650 R. X-ray given to immune ascites cells during transfer depresses the level of antibody produced later by 90 per cent or more. Thus the radiosensitivity of these immune peritoneal cells is similar to that of the reproductive capacity of human cells *in vitro* (Puck, Morkovin, Markus and Cieciura, 1957), and the competence of mouse spleen cells to give a primary or secondary antibody response after transfer (Makinodan, Kastenbaum and Peterson, 1962). Bacteriophage needs an X-ray dose higher by three orders of magnitude to lose only its infectivity (Epstein, 1953).

(2) (a) X-irradiation given to the host prior to immune ascites cell transfer does not decrease the amounts of antibody produced afterwards, but rather leads to a significant increase. Control groups of irradiated mice that received bacteriophage antigen alone at the same time after irradiation when cell transfer was carried out in the other groups showed that the capacity of the host mice to respond to a primary antigen stimulus was practically abolished for the duration of the experiment (Experiment IV) (cf. Makinodan and Gengozian, 1958).

(b) High levels of antibody are produced when immune peritoneal cells are injected into newborn mice within 20 hours after birth. Newborn mice are known to be immunologically relatively refractory towards antigen injections (e.g. Nossal, 1960), a statement that was confirmed in control mice in the present experiments.

Two conclusions can be derived from these experiments. First, free antigen in immunogenic quantities is not present in the system and the information on antibody specificity and the stimulus for antibody production are properties of the cells themselves. Secondly, the delayed antibody synthesis depends on the state of viability of the transferred cells.

The fact that the bulk of the antibody that appears after transfer is synthesized with a delay means that at the time of transfer the antibody-producing apparatus is not yet fully present, or not yet fully functional.

Unanswered remains the question whether the transferred cells differentiate themselves to become antibody producing cells (progenitor hypothesis) or whether they carry an agent other than antigen that transforms host cells into antibody-producing cells (transformation hypothesis). The progenitor hypothesis is favoured by the observation that the transferred cells themselves have to be alive for at least 4 days after transfer, that is, up to the time when actual antibody synthesis begins, as shown by transfer into homologous mice not pre-immunized against homograft antigens (Experiment I, T6). It is also favoured by current experiments employing millipore diffusion chambers (I. J. Weiler, to be published). Work is in progress to decide which of the various cell types in the population of peritoneal cells is the progenitor of antibody-producing cells.

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