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SECONDARY TETANUS ANTITOXIN RESPONSES IN MICE ELICITED PRIOR TO IRRADIATION*

by

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INTRODUCTION

The pioneer study by Benjamin and Sluka in 1908 on inhibition of antibody formation by X-rays revealed the importance of the temporal relationship between exposure to radiation and injection of antigen [1]. X-radiation delivered three days before injection of beef serum inhibited precipitin formation in rabbits. A similar exposure to X-rays delivered three days after injection of the antigen failed to repress production of specific antibody. These observations were partially confirmed in 1915 when Hektoen[2] reported inhibition of hemolysin production in the rat when X-radiation was delivered either before or after injection of sheep red cells. Although an absolute difference in radiosensitivity was not evident, the depressant effect of radiation on antibody production appeared to be less effective when radiation was given after injection of the antigen.

Craddock and Lawrence[3] observed that antibody formation was not reduced in rabbits when a radiation dose of 250 r was given four days after injection of typhoid vaccine and sheep red cells. They postulated the survival of reserve tissues after exposure to radiation with the capacity for antibody production. Kohn[4] reported that an X-ray dose of 175 r delivered three days before injection of sheep red cells effected a greater depression of the hemolysin response in rats than 600 r delivered 2 days after the antigenic stimulus. In studies concerning the temporal relationship of antigenic stimulus, and exposure to radiation, Dixon et al. [5] used I^{131}-labeled bovine gamma globulin and Taliaferro et al.[6] used sheep red cells to elicit primary and secondary antibody responses in rabbits. Both these groups reported an early radiosensitive phase of the antibody response, when antigen was injected during the first 12 hours after irradiation, and a relatively radioresistant phase, when antigen was given several days before irradiation.

Previous studies from this laboratory[7-8] have demonstrated complete suppression of primary tetanus antitoxin responses in mice when fluid toxoid was injected six hours to 21 days after exposure to 650 rep of gamma radiation. A corresponding repression of secondary antitoxin responses was obtained when the second injection of toxoid was given 30 minutes, 3 and 6 hours after exposure to 650 rep. The same radiation dose had little effect on antitoxin titer when radiation was delivered 10 days after a second stimulation (near the peak of the secondary response) and serum was obtained 10 days after radiation (20 days after antigenic stimulus), whereas, antitoxin formation was repressed when radiation was delivered 21 days after exposure to radiation (42 days after the antigenic stimulus). The present study is concerned with secondary tetanus antitoxin responses elicited in mice prior to short-term exposures of γ-radiation. Corresponding titers of antitoxin were obtained in irradiated and non-irradiated mice when radiation was delivered 4 days after the second injection of antigen. The incorporation of tritium activity into tetanus antitoxin when tritiated -L-histidine was injected at various times before and after exposure to radiation provides direct evidence for active antibody synthesis by surviving lymphoid tissues in irradiated animals when secondary antitoxin responses were elicited prior to irradiation with fluid tetanus toxoid.

MATERIALS AND METHODS

Animals and immunization procedure

Female Swiss albino mice were used in these experiments. These animals are specific pathogen-free and have been raised in our colonies as a pure strain since 1938. Excellent antibody responses have been elicited from these animals to the following particulate and soluble antigens: *Salmonella typhosa*,

Pseudomonas aeruginosa, type III pneumococcus, *Trichinella* antigen, influenza virus-type A and B, bovine serum albumin, human serum, chicken serum, ricin, botulinus toxoid, type A and B, and tetanus toxoid.

All mice were given a primary immunization when four weeks of age, consisting of a subcutaneous injection of 0.05 ml of aluminum phosphate-adsorbed purogenated tetanus toxoid (APTT), (Lederle). Secondary antitoxin responses were elicited with an intra-abdominal injection of 0.05 ml fluid tetanus toxoid (FTT) (Lederle). The intra-abdominal route of injection is superior in eliciting secondary antibody responses to either the subcutaneous or intravenous route of injection of the same antigen. The time of second antigenic stimulation in relation to time of exposure to gamma radiation is described for each experiment.

Serology and tetanus antitoxin titrations

All animals were sacrificed under ether anesthesia. The time of sacrifice is described with respective experiments. Heart blood was pooled from each group of animals and the serum titrated as a single specimen. The Ehrlich method of titration of antitoxin was used in these experiments. The tetanus toxin for titration of antitoxin contained 10^6 mouse M.L.D. per ml. The antigenic combining capacity of the toxin for tetanus antitoxin was compared to a standard obtained from the National Institutes of Health. One thousand M.L.D. were neutralized by 0.025 international units of tetanus antitoxin. A more detailed description of toxin-antitoxin titration procedures may be found in a previous report[7].

Cobalt-60- -radiation, calibration and dosimetry.

The radiation facility consisted of 40 slugs of cobalt-60 arranged to obtain a uniform field of exposure 16 inches below the source. The gamma-radiation dose of the circular (12.5 inch diameter) radiation area was measured with a Victoreen condenser R-meter and a Cobalt-60 type ionization chamber, 250 r full-scale deflection. The average dose rate based on a

series of 17 measurements in different quadrants was 13.9 r per minute, with a range in values from 12.5 to 15.0. The standard deviation from the mean was 1.05 r. Thirty to forty mice were irradiated at one time in a lucite box which was rotated at 2 rpm to provide uniform exposure to radiation. The mice were confined in the holder to a depth of 1 inch and constant aeration was delivered during the period of exposure. The exposure dose rate in r was converted to rad in tissue by the factor 0.96[9].

EXPERIMENTAL AND RESULTS

Effect of sublethal and supralethal radiation on antitoxin formation when radiation was delivered either before or after the second antigenic stimulus.

The experiment summarized in Table I was designed to compare the repressive effect of sublethal and supralethal radiation doses of 650 and 900 rads on secondary antibody responses when radiation was delivered either three hours before or four days after a second intra-abdominal injection of fluid toxoid. The non-irradiated animals in groups 1 and 2 served as controls of the amount of antitoxin produced during primary and secondary antitoxin responses. The second injection of toxoid was given 30 days after primary immunization. All animals were sacrificed for serum 10 days later. A radiation dose of 650 rads delivered three hours before the second stimulus (group 3) virtually abolished the secondary antibody response in these animals. The same dose of radiation did not repress antibody formation when it was delivered four days after the second stimulus (group 4). These animals produced more antitoxin per ml of serum than the non-irradiated control animals in group 2. A supralethal dose of 900 rads delivered three hours before the second antigenic stimulus to the animals in group 5 abolished the secondary antibody response and depressed the antitoxin titer to less than the titer obtained from primary immunization in non-irradiated animals (group 1). When a dose of 900 rads was delivered

four days after the second stimulus, the animals in group 6 produced 68% less antitoxin than corresponding non-irradiated control animals in group 2. Although supralethal radiation greatly repressed antibody formation, it is evident that these animals retained a limited capacity for synthesis of antitoxin when radiation was delivered four days after the second antigenic stimulus. If it is assumed that the antitoxin is synthesized only during the four-day period prior to the time of irradiation and released as circulating antibody, then one would not expect supralethal radiation to repress the titer of antitoxin to the relatively low value observed for group 6.

Effect of irradiation given at various times after the second antigenic stimulus.

The experiment shown in Table II was designed to test the repressive effect of radiation doses of 750 rads on the secondary antibody response when radiation was delivered six hours before and from one to seven days after the second injection of tetanus toxoid. All animals were given a primary stimulus of APTT 3 weeks prior to the time of the second stimulus. All animals were sacrificed and pooled sera obtained from each group ten days after the second stimulus of antigen. It should be noted that each group of mice had a different period of time for antibody formation in relation to the time of irradiation. The data show marked repression of secondary responses as compared to secondary responses obtained in non-irradiated mice (group 2) when radiation was delivered six hours before and 1, 2, and 3 days after the second stimulus. Significant titers of antitoxin were obtained in animals given 750 rads on day 4 to day 7 of the secondary response when serum was obtained from 6 to 3 days after radiation. Casual inspection of these data may tempt one to conclude that antibody formation is virtually completed by day 4 of the induction period and that antibody is merely released during the next few days of the secondary response.

Secondary antibody responses in irradiated and non-irradiated mice.

In the previous experiment, each group of animals had a different period of time for antibody formation in relation to the time of irradiation. The following experiment was carried out to compare the amount of antibody present each day in normal and irradiated mice when a dose of 800 rads was delivered four days after the second stimulus. The results are plotted in Figure 1 and each point on the curves represents a group composed of 30 mice. All animals received a second injection of fluid toxoid on day zero to elicit secondary responses. A radiation dose of 800 rads was delivered four days later. Groups of control and irradiated mice were sacrificed for serum four hours after radiation and daily through the following five days. The titers of antitoxin obtained in irradiated animals were only slightly less than the titers obtained in normal animals throughout the five-day post-radiation period. One may be tempted to conclude from these data that antibody synthesis was virtually completed by day 4 of the induction period and that antibody was merely released during the next few days. However, it should not be assumed that a dose of 800 rads completely stops antibody formation when the secondary response was elicited several days before irradiation. If the antibody is formed during the four-day period prior to irradiation and released as circulating antibody during the post-radiation period, one would expect irradiated and non-irradiated animals to have similar titers of antitoxin when serum is obtained at a time remote from the second injection of antigen.

The experiment illustrated in Table III was carried out to measure antibody titers in irradiated mice at an extended period of time. All animals received a primary immunization of APTT 39 days prior to the second antigenic stimulus. The titers obtained from animals in groups 1 and 2 served as controls of the amounts of antitoxin produced by non-irradiated animals during primary and secondary responses. Serum samples were obtained from each group of animals

9 days and 32 days after the second stimulus. As in previous experiments, the secondary response was severely repressed when the animals (group 3) were irradiated 3 hours before the second stimulus and serum obtained 9 days later. A slight increase in titer was observed when serum was obtained from these animals 32 days after the second stimulus. The animals in group 4, irradiated 4 days after the second stimulus, produced more antitoxin than corresponding non-irradiated control animals in group 2 when serum was obtained 9 days after the second stimulus. Even though the antitoxin titer was high 9 days after the second stimulus, it is evident that radiation delivered 4 days after the second stimulus repressed antitoxin formation in these animals during the following three weeks. If the radiation dose of 650 rads had completely stopped antibody formation in the animals of group 4, the titer of antibody 32 days after the second stimulus would be comparable to the titers observed in group 3 when sera were obtained at the same time. If the radiation dose delivered 4 days after the second stimulus was as effective in depressing antibody formation as the same dose given three hours before the second stimulus (group 3) the titer observed in group 4 would be of the order of 4 units of antitoxin. This assumption is based on a previous determination of a half-life of 4 days for isologous (same strain) antitoxin in our mice.

It was clearly evident in the preceding experiment that antibody continued to be released into the circulation 9 to 32 days after the second stimulus of antigen. Since antibody synthesis occurred when a dose of 600 rads was given 4 days after the second stimulus, an experiment was designed to determine if the antibody-forming mechanism in irradiated animals would respond to a third antigenic stimulus given shortly after exposure to radiation. The identity of the experimental groups and results obtained are summarized in Table IV. The non-irradiated animals in groups 1 and 2 served as controls of antibody

responses elicited by a second and third stimulus of antigen. Pooled serum samples were obtained from the animals in group 1 ten days after the second stimulus and from the mice in group 2 six days after the third stimulus. The data in Table IV indicate, as in previous experiments, that a dose of radiation delivered 30 minutes before the second stimulus effectively depressed the secondary antibody response (group 3). Comparable antibody responses were obtained in animals exposed to a dose of 600 rads four days after the second stimulus (group 4) and in non-irradiated animals in group 1. The animals in group 5 were irradiated 4 days after the second stimulus, or 30 minutes before the third antigenic stimulus. These animals produced 6.5 units of tetanus antitoxin as compared to 26.5 units of antitoxin produced by the non-irradiated controls (group 2). It is evident that a radiation dose of 600 rads delivered 4 days after the second stimulus failed to repress the secondary response (group 4), whereas the ability to respond to a third antigenic stimulus was severely repressed, when antigen was given 30 minutes after irradiation (group 5). These data clearly differentiate between the capacity of irradiated animals to give nearly normal secondary antitoxin responses and failure in their ability to respond to a third injection of tetanus toxoid.

Incorporation of tritium activity into antibody when radiation was delivered four days after antigenic stimulation.

The experiment diagrammed in Figure 2 and summarized in Table V was designed to determine if the antibody produced during the secondary response was synthesized during the four day period between the time of the second antigenic stimulus and the time of irradiation, or if antibody was actually produced and released as circulating antibody after exposure to 600 rads. All animals were given a primary antigenic stimulus of APTT two months prior to the time they were used in the experiment. The identity of the experimental groups composed of ten animals per group is shown in Table V. Each group of animals was given a

series of 4 injections of tritiated-L-histidine (H^3-H) either before or after the second injection of toxoid. The specific activity of the H^3-H was 1.7 C/mM. The H^3-H was diluted to 50 μc/ml and 5 μc per 0.1 ml was injected by intra-abdominal route. Each animal received 4 injections of H^3-H over a period of 2 days in a total dose of 20 μc of H^3-H. The second injection of 0.05 ml FTT was given by intra-abdominal route on day zero. A dose of 600 rads was delivered to the animals in groups 1 through 5, four days after antigenic stimulation. The animals in groups 6 through 10 served as non-irradiated controls. All groups of animals were sacrificed and pooled sera obtained 10 days after antigenic stimulation. The data shown in Table V clearly indicate that the animals in groups 1 through 5, irradiated 4 days after the second antigenic stimulus, produced antibody titers comparable to the titers observed in non-irradiated mice in groups 6 through 10. The animals in group 11 were not stimulated with antigen. Precipitin curves were determined for the various sera with 15 x concentrated fluid tetanus toxoid. The antibody in 0.4 ml samples of the sera was precipitated in the region of slight antigen excess with 0.25 ml of the concentrated toxoid. Subsequent tests of the supernatant fluid above the antigen-antibody precipitate indicated the presence of small amounts of tetanus toxoid and the absence of antibody. The antigen-antibody precipitates were washed five times in cold 0.85 percent saline, transferred to zinc planchets and dried for total tritium analysis with the gas counting procedures of Christman[10].

The activity of tritium incorporated into antibody which was precipitated with specific antigen is shown in Table V. The values are given in disintegrations per minute (dpm) per ml of serum. The last column in Table V summarizes the activity of tritium present in the fluid above the antigen-antibody precipitate; presumably the tritium activity may be present either as free amino acid, or as metabolic products of the tritium-labeled histidine, or as non-antibody

serum proteins. The animals in group 11 were not given primary and secondary antigenic stimulations. These animals served as controls of the amount of tritium activity present in normal serum when serum was obtained 9 days after the last injection of H^3-H. The observed value of 3,890 dpm per ml of serum (group 11) was 42 and 30 percent less than the total values observed for precipitated antibody and non-precipitated fluid in groups 2 and 7 respectively. The activity counted in the antigen-antibody precipitates are also plotted in Figure 2. These data clearly demonstrate that minimal amounts of tritium activity were incorporated into antibody when H^3-H was injected on day 2 and day 1 before injection of the antigen and on days 1, 2, and 3 after the second injection of antigen. However, a marked increase in incorporation of tritium activity occurred when the labeled-amino acid was injected on days 4 and 5 and on days 6 and 7 after the second antigenic stimulus. Moreover, when the first injection of H^3-H was given one hour after exposure to 600 rads on day 4 and on day 5, tritium activity was incorporated into antibody to levels as high as was observed in non-irradiated animals. A corresponding incorporation of tritium activity into antibody was found for irradiated animals when H^3-H was injected on days 6 and 7 after antigenic stimulation (days 2 and 3 after exposure to 600 rads). It is evident from these data that the antitoxin produced during the secondary antibody response was not preformed during the induction phase of the response and merely released on day 4 following the second antigenic stimulus. It appears that there are cells capable of synthesizing antibody in the irradiated animals since the antibody produced by these animals contained significant amounts of tritium activity. It should be emphasized that the animals in groups 1 through 5 produced antibody titers comparable to the titers observed in non-irradiated mice. If the antibody elicited by the second antigenic stimulus were synthesized only during the induction phase, we might expect a marked incorporation of tritium activity into antibody when the labeled amino acid was

injected during the first 3 days after the second stimulus of antigen in groups 2 and 3 and 7 and 8. The observed incorporation of tritium activity into antibody when the initial injection of H^3-H was given 4 to 6 days after the second stimulus (one hour to 2 days after irradiation) clearly demonstrated synthesis of antibody after exposure to 600 rads. It is obvious that antibody-forming cells are capable of producing specific antitoxin after exposure to radiation when antigenically stimulated 4 days prior to radiation. However, these irradiated mice gave reduced antitoxin responses when a third injection of antigen was given 30 minutes after exposure to 600 rads.

DISCUSSION

Conclusive evidence has appeared concerning the effect of the temporal relationship between exposure to ionizing radiation and injection of antigen on repression of antibody responses. There appears to be general agreement that radiation delivered before injection of an antigen is more effective in depressing antibody formation than an equal or greater dose of radiation given 2-5 days after injection of antigen. It is difficult to evaluate the discrepancies in results reported by different investigators since a wide variety of antigens, time relationships between exposure to radiation and antigenic stimulus, total radiation dose and laboratory animal species have been used in these studies. An excellent discussion of these findings may be found in a recent paper by Simic and Sljivic[11].

The studies of Dixon et al.[5] and Talisferro et al.[6] introduced the concept of a limited radiosensitive phase (adapting phase) of the antibody response when radiation was given before the antigen and a later radioresistant production phase when radiation was delivered after injection of the antigen. Many investigators have used this concept to evaluate and compare their results to the findings from other laboratories. It should not be assumed that the radioresistant production phase of the antibody response is completely insensitive

to radiation. Repression of the secondary antitoxin response was previously reported when radiation was delivered 21 days after the second antigenic stimulus and serum was obtained 21 days after exposure to radiation[7]. In the present study, irradiated animals produced as much antitoxin as non-irradiated animals when a radiation dose of 650 rads was delivered 4 days after the second injection of toxoid and serum was obtained 5 days later. A radiation-induced repression of the secondary response in these animals was observed when serum was obtained 32 days after the second injection of antigen (Table III). Irradiated animals showed nearly normal secondary antitoxin responses when a radiation dose of 600 rads was delivered 4 days after the second stimulus and failed to respond to a third antigenic stimulus delivered 30 minutes after exposure to radiation (Table IV). From these data it is possible to clearly differentiate between the capacity of irradiated animals to produce nearly normal secondary antibody responses and the inability of the same animals to respond to a third antigenic stimulus. These findings also demonstrate the radiosensitive nature of the secondary response when sera were obtained remote from the time of the second antigenic stimulus and exposure to radiation.

Simic and Sljivic[11] reported all three phases of the hemolysin response to sheep red cells in rats as radiosensitive. They interpreted the radiosensitivity of the pre-induction phase, the induction phase, and the production phase of the antibody response as a result of interference with different processes involved in the synthesis of antibody. Modification of the temporal relationship between injection of antigen and irradiation resulted in radiation injury either to one phase only or a multiple radiation-induced injury to all three phases. They proposed that irradiation affects antibody synthesis both through inhibition of nucleic acid biosynthesis and proliferation of antibody-forming cells. Fitch et al.[12] demonstrated a close correlation between hyperplasia and increase in pyroninophilic cells in the spleen and antibody

formation following intravenous stimulation with sheep red cells in the rat. X-irradiation abolished the normal pattern of cellular reaction to antigenic stimulation. In a later study[13] a depression of the peak titer of hemolysin antibody was observed when radiation was delivered 4 days after stimulation with the antigen. The depression of circulating antibody appeared to be related to alteration of the histologic response following antigen stimulation.

In a recent study in mice immunized with bovine serum albumin or tetanus toxoid, Cottier and Jost[14] have shown with in vivo labeling with tritium-labeled cytidine, and tritium-labeled thymidine that most of the lymphatic organs of immunized animals have a greater percentage of small lymphocytes actively synthesizing RNA than that found in non-immunized mice. On the other hand, the relative radioresistance of a particular lymphatic organ paralleled its content in small lymphocytes actively synthesizing RNA and also paralleled its content in small lymphocytes that have formed a greater cytoplasmic mass and more granular endoplasmic reticulum. The values obtained with H^3-cytidine labeling were subtracted from the values obtained with H^3-thymidine labeling since the latter goes only to DNA. A greater percentage of markedly H^3-cytidine labeled small lymphocytes survived than weakly labeled or non-labeled cells when tested three hours after a short-term radiation dose of 200 rads. The labeling index and mean grain count per labeled intact lymphocytes was higher in immunized, irradiated animals than in immunized non-irradiated controls. Plasma cells and their immediate precursors tolerate even higher radiation doses; these cells may be found morphologically intact in appreciable numbers after short term exposure to 950 rads. However, Cottier and Jost emphasized that their study was limited to small lymphocytes and although they may be of importance, the small lymphocytes may not necessarily be precursors of cells involved in antibody formation. The origin of the large lymphoid cells

proliferating during secondary antibody responses is still in doubt. In the present study, the nearly normal secondary responses of mice irradiated on days 4 to 7 may possibly result from antibody formation by radioresistant cells that have proliferated in response to the second injection of antigen administered prior to irradiation. The observed inability of these irradiated mice to respond to a third injection of antigen may be accounted for either by failure to accept antigen, as proposed by Kohn[4], or by radiation-repressed proliferation of a new and more radiosensitive group of cells ultimately concerned with antibody synthesis.

The present observation of incorporation of tritium activity into tetanus antitoxin when the initial injection of tritiated-histidine was given 4-6 days after the second stimulus (1 hour to 2 days after radiation) clearly demonstrated synthesis of antibody in mice after exposure to 600 rads. It is apparent that antibody-forming cells were present in these animals capable of producing specific antitoxin after exposure to radiation when animals were antigenically stimulated 4 days prior to radiation. It is evident from the data shown in Table V and plotted in Figure 2 that the antitoxin produced in both irradiated and non-irradiated animals during the secondary antitoxin response was not pre-formed during the induction phase of the response, and merely released on day 4 or day 5 following the second antigenic stimulus.

Convincing evidence has been brought forth by Gros et al.[15] indicating de novo synthesis of antibody molecules. Heidelberger et al.[16] demonstrated a rapid appearance of radioactivity in normal components of rabbit plasma after feeding N^{15}-labeled glycine. Passively transferred anti-pneumoccocal rabbit serum did not incorporate radioactivity from the N^{15}-labeled amino acid to any extent. Bulman and Campbell[17] administered C^{14}-labeled leucine to rabbits that had been passively immunized and found that while C^{14} activity appeared in the normal serum constituents, the passively transferred antibody did not

incorporate C^{14} following administration of the labeled amino acid. Green and Anker[18] concluded that none of the major components of the plasma proteins (albumin, non-antibody gamma globulin, and remaining globulins) was a direct intermediate in the incorporation of C^{14}-labeled glycine into antibody protein. Their work indicated that most of the antibody protein is formed by de novo synthesis from amino acids injected after administration of the antigen. Askonas and White[19] observed incorporation of C^{14} activity into anti-ovalbumin in guinea pigs when C^{14}-labeled glycine was added to cultures of lymph node and other lymphoid tissues. After an initial lag period of20-30 minutes, the rates of incorporation of C^{14} activity into both anti-ovalbumin and gamma globulin fractions remained steady over a period of 2 hours and then rapidly diminished.

Taliaferro and Talmage[20] found very little incorporation of S^{35}-labeled amino acids during the induction period when recipient rabbits received spleen cells from donors injected with S^{35}-labeled amino acids and recipients received the same labeled amino acids only during the production phase of the antibody response. Their results indicated that most of the sulphur activity present in precipitating antibody was drawn from the amino acid pool during the rise of serum antibody and not incorporated during the first three days of the induction period after injection of bovine serum albumin. They suggested that the rate of rise of serum antibody reflected the rate of antibody formation and that the induction period is concerned with production of antibody-synthesizing enzymes rather than with amino acid antibody precursors. In a later study, Taliaferro and Taliaferro[21] studied the transit time of S^{35}-labeled amino acids in rabbits during the secondary response to bovine serum albumin. Activity from S^{35} did not appear in the precipitins at 20 minutes, but was present in significant amounts at 40 minutes and reached a plateau at 45 hours when the S^{35}-labeled amino acids were given during the rise of the secondary response 3-4 days after antigenic stimulation.

The data presented herein clearly indicate that significant amounts of tritium activity were not found in tetanus antitoxin (precipitated with tetanus toxoid in slight antigen excess) when H^3-L-histidine was injected on day 2 and day 1 of the pre-induction period or during the first three days of the induction period following the second antigenic stimulus. However, a marked increase in incorporation of tritium activity occurred when the labeled-amino acid was injected on days 4 and 5 and on days 6 and 7 after the second antigenic stimulus. Moreover, the tritium activity incorporated into antibody was as high in irradiated animals as in non-irradiated control animals when the first injection of H^3-H was given one hour after exposure to 600 rads on day 4 and, two days after irradiation on day 6. Since the labeled amino acid was administered in 4 injections to each group of animals over a period of 2 days, it was not possible to determine the transit time of the injected labeled amino acid from circulating blood into antibody. Nonetheless, it is evident that the great increase in antitoxin observed during the secondary antibody responses in irradiated and non-irradiated animals was not preformed during the induction phase of the response and merely released on day 4 to day 10 during the secondary response. These data indicate the presence of cells in irradiated animals capable of synthesizing antibody since the antibody produced by these animals and precipitated _in vitro_ by specific antigen contained significant amounts of tritium activity. Although a radiation dose of 600 rads delivered 4 days after the second stimulus failed to repress the secondary response as shown in Table IV, the ability to respond to a third antigenic stimulus was severely repressed. These findings indicate that antibody-producing cells or their precursors have proliferated in response to the second antigenic stimulus and survived long enough after irradiation to produce nearly normal secondary tetanus antitoxin responses. It is not known at this time if failure to respond to the third injection of antigen was due to an inability to accept antigen

when toxoid was injected 30 minutes after irradiation. The possibility should also be considered that a new and different radiosensitive group of immunologically competent cells may fail to proliferate and synthesize antibody.

SUMMARY

1. Secondary tetanus antitoxin responses were abolished in mice when sublethal radiation doses of 650 rads were delivered by short-term exposure three hours before the second injection of antigen. Nearly normal secondary responses were observed when the same radiation doses were delivered four days after antigenic stimulation, and sera were obtained 6 days later.

2. Radiosensitivity of the seemingly radioresistant secondary antibody responses was demonstrated by ultimate repression of antitoxin titers when radiation was delivered four days after antigenic stimulation and sera were obtained four weeks after irradiation (32 days after the second injection of toxoid).

3. It was possible to clearly differentiate between the capacity of these irradiated animals to produce nearly normal secondary responses and failure of the same animals to respond to a third antigenic stimulus when radiation was delivered four days after the second stimulus, and a third injection of antigen was given 30 minutes after the single exposure to 650 rads.

4. A marked incorporation of tritium activity appeared in antitoxin produced during secondary responses of irradiated and non-irradiated mice when tritium-labeled L-histidine was injected on days 4 and 5 and on days 6 and 7 after the second stimulus of tetanus toxoid. The data indicate that the antibody produced during secondary responses in irradiated and non-irradiated mice was not preformed during the induction phase and merely released on days 4 or 5

following the second stimulus of antigen. These findings indicate the presence of antibody-producing cells or their precursors that have proliferated in response to the second antigenic stimulus and survived long enough after irradiation to produce nearly normal secondary tetanus antitoxin responses.

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FIGURE LEGEND

1. Showing secondary responses obtained daily in 12 groups of mice when radiation was delivered 4 days after antigenic stimulation. Each point on the curves represents a group of 30 mice.

2. Showing tritium radioactivity counted in antibody precipitated from the above secondary response sera. Each point on the curves represents a group of 10 mice. Tritium-labeled-L-histidine (H^3-H) was injected during the periods indicated.

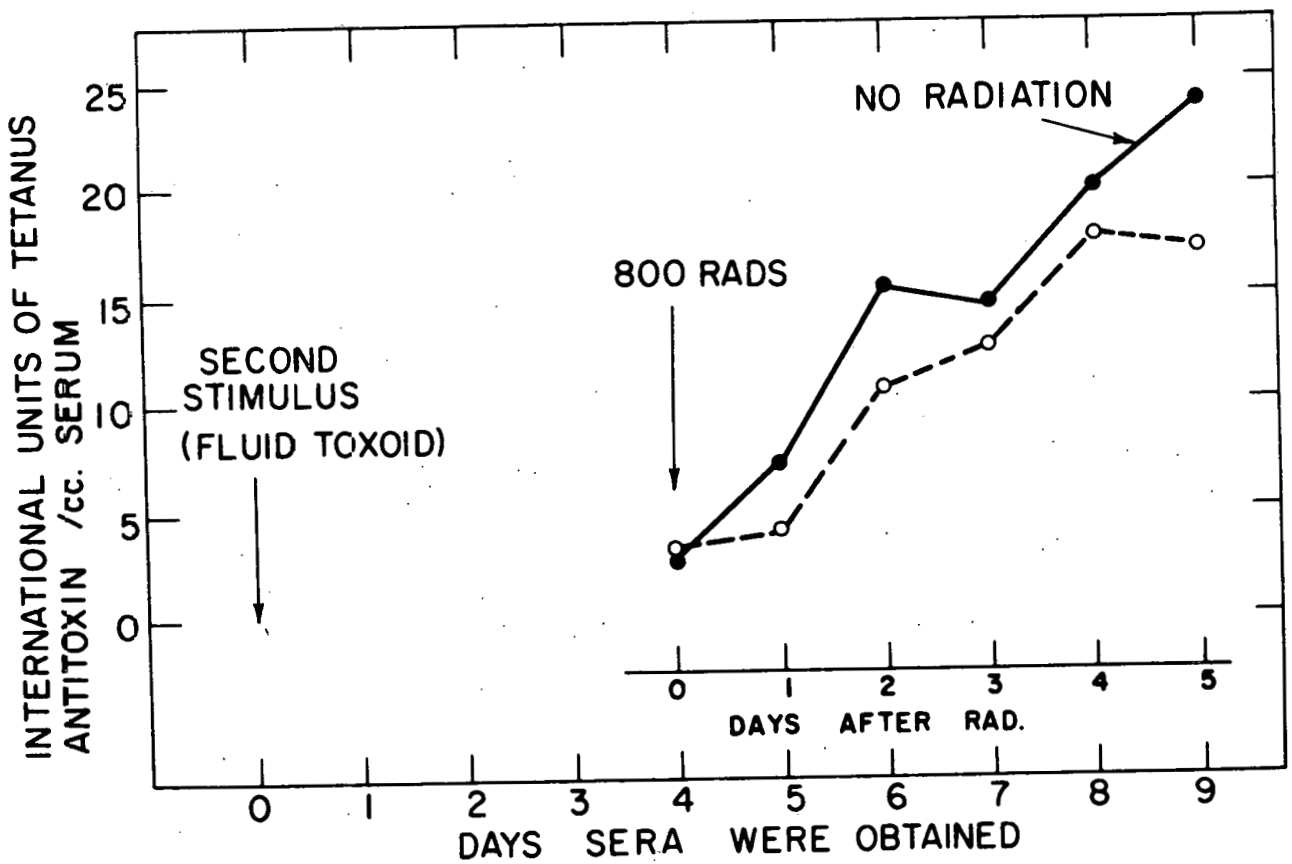
INTERNATIONAL UNITS OF TETANUS ANTITOXIN /cc. SERUM
25
20
15
10
5
0
SECOND STIMULUS (FLUID TOXOID)
800 RADS
NO RADIATION
0 1 2 3 4 5
DAYS AFTER RAD.
0 1 2 3 4 5 6 7 8 9
DAYS SERA WERE OBTAINED

FIGURE 1

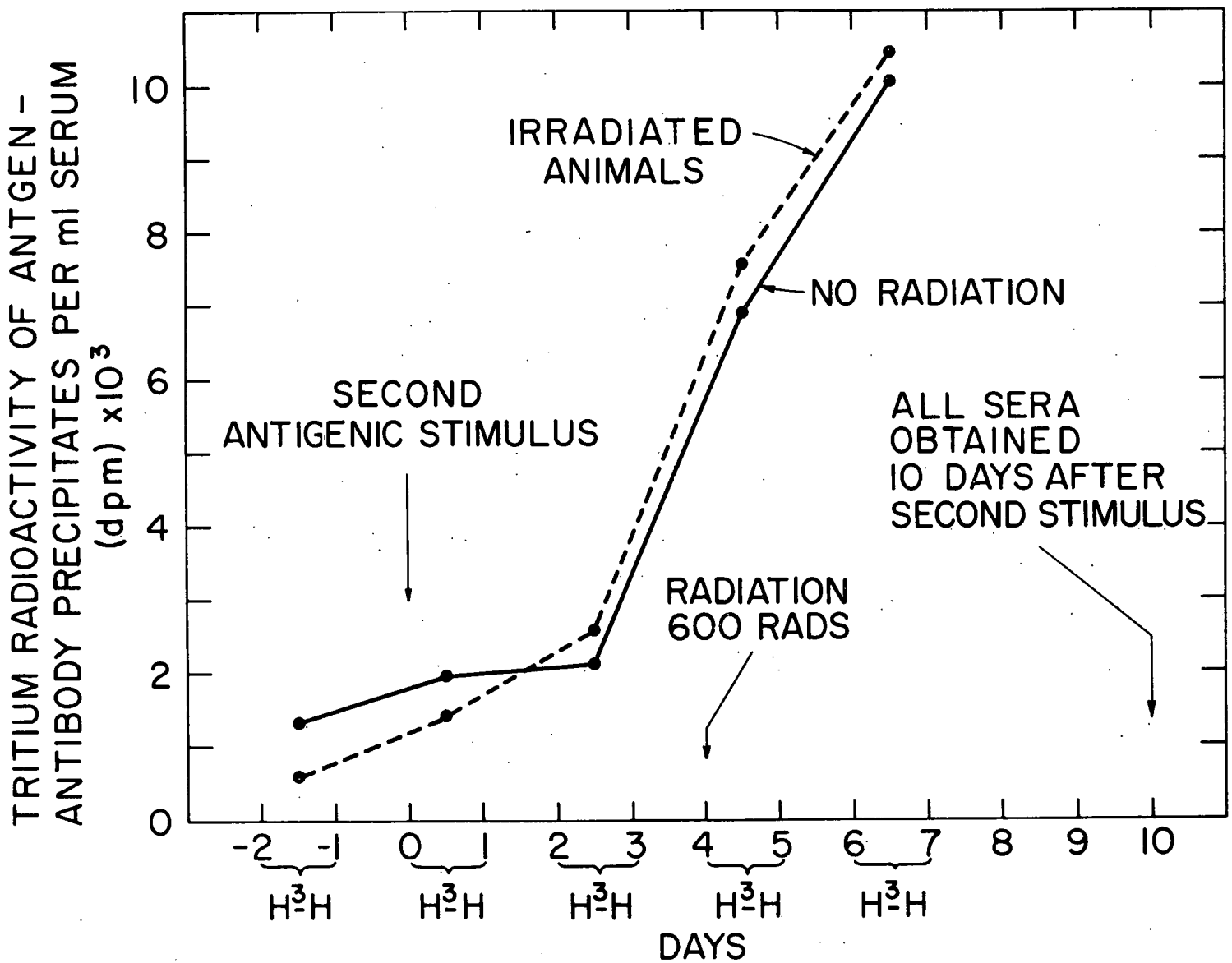
TRITIUM RADIOACTIVITY OF ANTGEN-ANTIBODY PRECIPITATES PER ml SERUM (dpm) x10³
0
2
4
6
8
10
IRRADIATED ANIMALS
NO RADIATION
SECOND ANTIGENIC STIMULUS
ALL SERA OBTAINED 10 DAYS AFTER SECOND STIMULUS
RADIATION 600 RADS
-2
-1
0
1
2
3
4
5
6
7
8
9
10
H³-H
H³-H
H³-H
H³-H
H³-H
DAYS

FIGURE 2

TABLE I

EFFECT OF SUBLETHAL AND SUPRALETHAL γ-RADIATION ON ANTIBODY FORMATION WHEN RADIATION WAS DELIVERED FOUR DAYS AFTER ANTIGENIC STIMULATION

Group No.	Number of Mice per Group	Whole Body γ-Irradiation (Rads)	Time of Irrad. in Relation to Second Stimulus	Second Stimulus FTT (i.a.)	Tetanus, Antitoxin Int.Units/ml Serum
1	105	None	Not given	No	0.35
2	105	None	Not given	Yes	18.25
3	105	650	3 hrs before	Yes	0.93
4	105	650	4 days after	Yes	20.00
5	35	900*	3 hrs before	Yes	0.075
6	35	900	4 days after	Yes	5.84

* In a control group given 900 rads, 34 of 35 mice died within 30 days.

TABLE II

EFFECT OF RADIATION DELIVERED AT DIFFERENT TIMES ON SECONDARY ANTITOXIN RESPONSES

Group No. (35 mice per group)	γ-Radiation Dose (Rads)	Second Stimulus Tetanus Toxoid	Time of Radiation in Relation to Second Stimulus	Antitoxin International Units/ml Serum*	Time Sera Obtained in Relation to Time of Irradiation	No. Died Post-Radiation
1	None	None	-	0.04	-	0
2	None	Yes	-	16.7	-	0
3	750	Yes	6 hrs. before	0.05	10 days	7
4	750	Yes	1 day after	0.08	9 days	9
5	750	Yes	2 days after	1.2	8 days	5
6	750	Yes	3 days after	4.15	7 days	9
7	750	Yes	4 days after	14.57	6 days	7
8	750	Yes	5 days after	12.5	5 days	1
9	750	Yes	6 days after	12.5	4 days	0
10	750	Yes	7 days after	10.0	3 days	0

* All animals sacrificed for serum 10 days after the second injection of tetanus toxoid.

TABLE III

SECONDARY ANTIBODY RESPONSES WHEN SERA WERE OBTAINED NINE AND THIRTY-TWO DAYS AFTER THE SECOND ANTIGENIC STIMULUS

Group No.* (35 mice/group)	Time of Radiation Dose (650 Rads)	Second Stimulus FTT (i.a.)	Tetanus Antitoxin International Units/ml pooled Serum	
			Sera Obtained 9 days After Second Stimulus	Sera Obtained 32 Days After Second Stimulus
1	None	None	0.48	0.53
2	None	Yes	15.9	22.0
3	3 hrs before second stimulus	Yes	1.0	3.1
4	4 days after second stimulus	Yes	25.5	12.0

* All animals received a primary antigenic stimulus of 0.05 ml. adsorbed toxoid by subcutaneous route.

TABLE IV

ANTIBODY RESPONSES IN MICE IRRADIATED FOUR DAYS AFTER A SECOND ANTIGENIC STIMULUS WHEN A THIRD ANTIGENIC STIMULUS WAS GIVEN THIRTY MINUTES AFTER IRRADIATION

Group No.* (35 mice/group)	Time of Exposure to 600 Rads in Relation to Second Antigenic Stimulus	Second Stimulus FTT (i.a.)	Third Stimulus 30 Minutes After Irradiation	Time Sera Obtained After Antigenic Stimulation	Tetanus Antitoxin International Units/ml Pooled Sera	Antitoxin Produced After Third Stimulus
1	None	Yes	None	10 days after second	14.8	-
2	None	Yes	Yes	6 days after third	41.25	26.45
3	30 min. before	Yes	None	10 days after second	1.5	-
4	4 days after	Yes	None	10 days after second	15.5	-
5	4 days after	Yes	Yes	6 days after third	22.0	6.5

* All animals received a primary antigenic stimulus of 0.05 ml. adsorbed toxoid by subcutaneous route.

TABLE V

INCORPORATION OF TRITIUM FROM TRITIUM-LABELED L-HISTIDINE INTO MOUSE TETANUS ANTITOXIN

Group No. (10 mice per group)	Time of Injection H^3-Histidine in Relation to Second Stimulus of Antigen* (Days)	γ-Radiation Given Day 4 After Injection of Antigen (Rads)	Tetanus Antitoxin International Units/ml	dpm/ml in Antibody Precipitated with Antigen	dpm/ml of Fluid Above Ag-Antibody Precipitate
1	-2, - 1 Before	600	20.0	677	887
2	1 hour, 1 After	600	21.3	1420	5280
3	2, 3 After	600	19.4	2570	1050
4	4, 5 After	600	20.0	7590	1210
5	6, 7 After	600	20.0	10430	8365
6	-2, - 1 Before	None	18.8	1320	-
7	1 hour, 1 After	None	20.0	1997	3450
8	2, 3 After	None	18.8	2245	3950
9	4,5 After	None	20.0	6815	1345
10	6,7 After	None	21.3	10052	8760
11	0, 1	(No Antigen) None	None	None	3890

* Second antigenic stimulus of 0.05 ml fluid tetanus toxoid injected by intra-abdominal route on day zero.