

## GAMMA GLOBULIN AND ANTIBODY FORMATION IN VITRO\*

### VI. EFFECT OF X-IRRADIATION ON THE SECONDARY ANTIBODY RESPONSE IN VITRO‡

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Studies on the effect of X-irradiation on antibody formation in vivo have clearly established that X-irradiation is much more inhibitory to antibody formation when given before as opposed to after antigen (1-3). This seems to imply that cells which are actually synthesizing antibody are more radioresistant than cells which have the potential to respond to antigenic stimulation. However, this difference might be only apparent in view of the complexity of factors involved in studying the effect of irradiation in the intact animal. Many antigens are known to produce nonspecific stimulation of the reticuloendothelial system (RES) and might thereby protect against the effect of irradiation given subsequently (4). A high level of circulating antibody during the productive phase might mask a depression of the rate of antibody synthesis (5). It is known that at a certain stage of the immune response X-irradiation can actually enhance the amount of antibody produced (6). Such an enhancement may be due to an increased population of antibody-forming cells and may prevent precise evaluation of a possible simultaneous inhibitory effect on individual antibody-forming cells.

Another interesting phenomenon is the progressive decrease in the capacity to respond to antigen over the first 6-48 hr after X-irradiation. This has been consistently observed for the primary response (1) and in a few cases also for the secondary (7-9). This suggests that cells which have suffered irradiation

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damage can be protected by early exposure to antigen. Such a prevention or repair of irradiation damage in vivo might occur in the lymphoid tissue itself or be mediated by other tissues.

Many of the difficulties encountered in the interpretation of in vivo effects of X-irradiation may be circumvented by the use of an in vitro system. Another advantage of an in vitro study is that the period of exposure to antigen can be more readily manipulated. A method of in vivo culture allowing precise quantitation was used effectively by Makinodan et al. (10). With this system no significant difference could be found when the sensitivity of primed and normal cells to X-irradiation were compared.

At present, a completely in vitro system is not readily available for the study of irradiation effects on various phases of the primary response. The study of the secondary response in vitro as described by Michaelides et al. (11) is much more amenable to a critical evaluation of such effects. This method has already been successfully employed in the analysis of the mechanism of action of various immunosuppressant drugs on antibody formation (12, 13). In the following experiments the influence of radiation given at various times before or after secondary antigenic stimulation in vitro will be analyzed and compared with the effect of certain drugs.

#### *Materials and Methods*

*Animals.*—Adult male New Zealand white rabbits were used in all experiments. Each animal was immunized with a single dose of 10 mg bovine serum albumin (BSA), and/or 120 Lf diphtheria toxoid (DT) injected into the hind foot-pads. The popliteal lymph nodes were used for tissue culture 6–18 months later. Injections of 0.5 ml of a 20% sheep erythrocyte (SE) suspension intravenously, and of 0.25 ml in each hind foot-pad were also used to immunize rabbits. Popliteal lymph nodes from these animals were cultured at intervals from 10 to 210 days. In all cases a serum sample was taken on the day of lymph node excision.

*Antigens and Immunosuppressants.*—Diphtheria toxoid (DT) containing 1200 Lf/ml was supplied through the courtesy of Mr. W. S. Hammond of Lederle Laboratories, Pearl River, N. Y.

A standard dose of 1 Lf/ml of culture medium was used to elicit a secondary response.

Bovine serum albumin (BSA), purchased in crystalline form from Armour Pharmaceutical Co., Kankakee, Ill. was employed at a concentration of 50  $\mu$ g/ml culture medium.

In the case of tissue sensitized to sheep erythrocytes,  $10^9$  red cells were used for the in vitro secondary antigenic stimulation.

All antigens were allowed to remain in the culture medium for 6–12 hr. After removal of the antigen the tissue was thoroughly washed with several volumes of Hanks' balanced salt solution.

5-Bromodeoxyuridine (BUDR), A grade, was obtained from Calbiochem, Los Angeles, and used at a concentration of 250  $\mu$ g/ml of medium.

Colchicine, USP, purchased from Amend Drug and Chemical Co., Inc., N. Y., was employed at a concentration of 0.4  $\mu$ g/ml. All solutions were sterilized by Seitz filtration.

X-Irradiation was given via a Picker Therapeutic unit, run at 220 kvp and 20 ma, at a distance of 20.5 cm from the tissue. Inherent filtration of the tube was the glass equivalent of 0.25 mm Cu and oil equivalent of 1.0 mm Al. External filtration included 0.5 mm Cu and 1.0 mm Al. The dose delivered was approximately 130 R/min giving a total dose of 450–500 R.

*Tissue Culture Methods.*—The popliteal lymph nodes were cut into 1–2 mm fragments and approximately 20 mg (wet weight) of tissue was distributed over the walls of 16 × 125 mm screw-cap roller type culture tubes, previously coated with normal rabbit plasma. To each culture tube was added 1 ml of a modified Eagle's medium containing 25% normal rabbit serum (11). Within each experiment, duplicate tubes were prepared for each time interval. Culture tubes were kept in a 37°C incubator. Medium was replaced every 3–4 days and immediately following X-irradiation. Aseptic technique was carefully observed throughout these studies.

*Antibody Titrations.*—Culture fluids and sera were stored at –20°C until they were titrated for antibody. Agglutination titers were determined using sensitized tanned sheep erythrocytes (14) or simple hemagglutination. Sensitivity of antibody to 0.1 M mercaptoethanol treatment was also determined (15).

*Radioautography of Immuno-electrophoretic Patterns.*—Tissues were cultured for 24 hr in a modified Eagle's medium containing 0.5% ovalbumin and 1  $\mu$ c each of <sup>14</sup>C-lysine (600–1500  $\mu$ c/mg) and <sup>14</sup>C-isoleucine (675–2000  $\mu$ c/mg) per ml. The resultant tissue culture fluids were dialyzed against 0.015 M pH 7.2 phosphate-buffered saline, concentrated by lyophilization, and analyzed by means of radioautography of immuno-electrophoretic patterns (16). Since the culture fluids contained only minute amounts of the individual labeled serum proteins, normal rabbit serum was used as a carrier. The microimmuno-electrophoretic patterns were developed using sheep anti-whole rabbit serum.

## RESULTS

*Effect of X-Irradiation at Different Intervals after Exposure to Antigen.*—In this series of experiments sensitized lymph node fragments were distributed over tissue culture tubes and then immediately (day 0) reexposed to either DT alone or to both DT and BSA. Two types of controls were used. Some tubes received antigen but no irradiation, and others received neither. A few tubes were exposed to 500 R X-irradiation immediately after receiving antigen, a second group of tubes was exposed to X-ray on day 2 or 3, and a third group on day 6. A few tubes received no antigen but were irradiated either on day 0 or day 3.

The results are summarized in Figs. 1 and 2. It can be seen that X-irradiation on day 6 did not significantly affect the production of antibody to either DT or BSA. Irradiation on day 2 or 3 slightly reduced the level of antibody production attained, whereas irradiation on day 0 produced a marked depression of antibody production in these experiments (Fig. 1). Tissue which did not receive antigen never showed antibody production to DT (Fig. 1), but exhibited a significant response to BSA (Fig. 2). The culture fluid on day 3 contained much less antibody than the medium removed on day 6, indicating a development of antibody production *in vitro*, rather than a continuation of activity already present *in vivo*. Like the response induced by adding antigen on day 0, this spontaneous anti-BSA production was reduced to a greater extent by irradiation on day 0 than on day 3 (Fig. 2).

The anti-BSA and anti-DT production continued in irradiated tissue for 2–3 wk. At the end of these experiments (3 wk) the medium was replaced by a medium containing <sup>14</sup>C-amino acids in order to study the  $\gamma$ -globulin production

in these tissues. At this late stage in the experiments, all tissues, regardless of treatment, exhibited a residual ability to incorporate the labeled amino acids into  $\gamma$ -globulin.

*Effect of X-Irradiation at Different Intervals Before Exposure to Antigen.*— In this second series of experiments lymph node fragments from rabbits immunized with DT or SE were first irradiated with 450-500 R and subsequently reexposed to DT or SE.

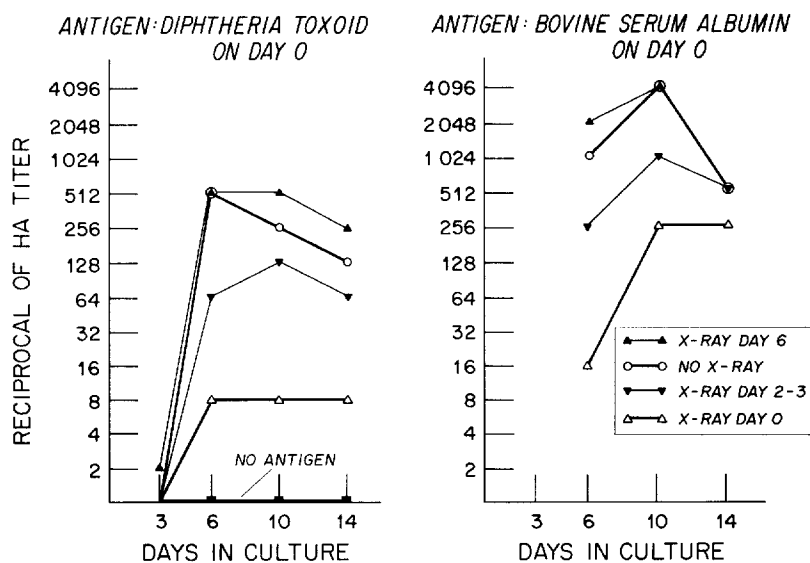


FIG. 1. Average hemagglutination (HA) titers of culture fluids removed every 3-4 days from tissue subjected to radiation (500 R) at varying intervals after secondary exposure to antigen in vitro. Results of three experiments (six culture tubes per point) are shown in the case of DT-sensitized lymph nodes, and of two experiments (four tubes) in the case of BSA-sensitized lymph nodes.

Fig. 3 illustrates the results obtained with DT-sensitized lymph nodes. Non-irradiated tissue which received the antigen within 8 hr after initiation of the culture period produced a little more antibody (average peak titer 1/256) than tissue which received the antigen at 12-24 hr (average peak titer 1/128). Delay of exposure to antigen in the case of irradiated tissue resulted in a much greater depression of antibody production. An average peak titer (34 tubes) of 1/32 was produced by tissue exposed to DT within 0-8 hr after X-ray (Fig. 3). This was a somewhat less pronounced reduction than was found in the previous series of experiments (Fig. 1). A delay of 12 hr after irradiation before re-exposure to DT resulted in a greater inhibition (average peak titer 1/8), and a delay of 24 hr completely inhibited the antibody response in all tubes (Fig. 3). Tissue not reexposed to DT never produced detectable anti-DT.

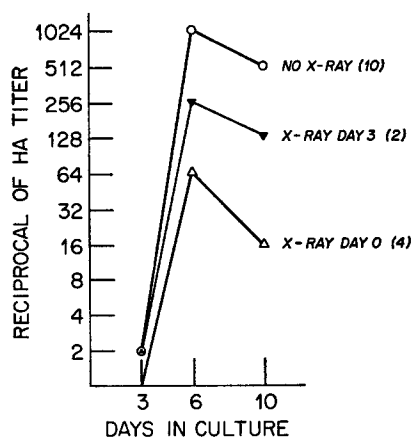


FIG. 2. Average hemagglutination (HA) titers of culture fluids removed every 3-4 days from lymph node cultures which had not been reexposed to antigen in vitro. Radiation was given immediately or 3 days after the tissue was put in culture. The numbers in parentheses following the line labels indicate the number of culture tubes used in each experimental group.

Some additional cultures were used for the study of  $\gamma$ -globulin synthesis in these tissues.  $^{14}\text{C}$ -amino acid containing medium was added on day 4 of the culture period and left with the tissue for 24 hr. Some incorporation of the amino acids into  $\gamma$ -globulin could be shown even in the case of tissue which exhibited no detectable antibody response, but tissue with a high level of antibody production showed greatly increased production of  $\gamma$ -globulin. X-irradiation immediately followed by antigen on the first day of culture did not prevent the induction of enhanced levels of  $\gamma$ -globulin synthesis. When

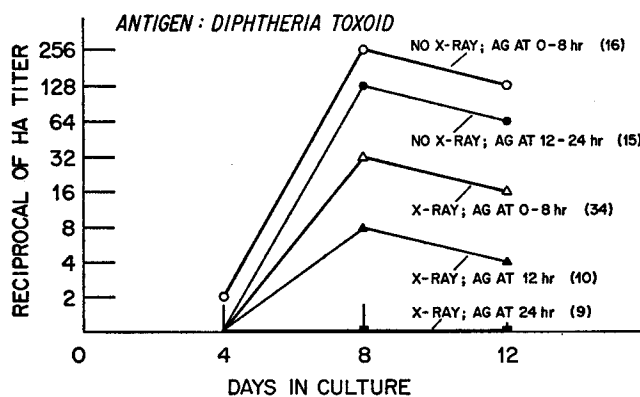


FIG. 3. Average hemagglutination (HA) titers of culture fluids removed every 4 days from tissue receiving a secondary exposure to antigen (AG = diphtheria toxoid) in vitro at varying intervals after radiation (450-500 R). The numbers in parentheses following the line labels indicate the number of culture tubes used in each experimental group.

the antigen was given 24 hr after irradiation, however, this increase in  $\gamma$ -globulin synthesis was not observed.

Fig. 4 shows the results obtained with lymph nodes from animals immunized with SE 2-4 wk previously. A delay of 24 hr before reexposure to SE had no detectable effect on the peak titers obtained with nonirradiated tissue, but the effect of a delay in the reexposure to SE in the case of irradiated tissue was similar to that observed with DT-sensitized lymph nodes. A delay of 12-24 hr was again more inhibitory than a delay of 0-8 hr, but it should be noted that the response was not completely eliminated even with a delay of 24 hr (Fig. 4). This may mean that the secondary response to SE is slightly more radio-

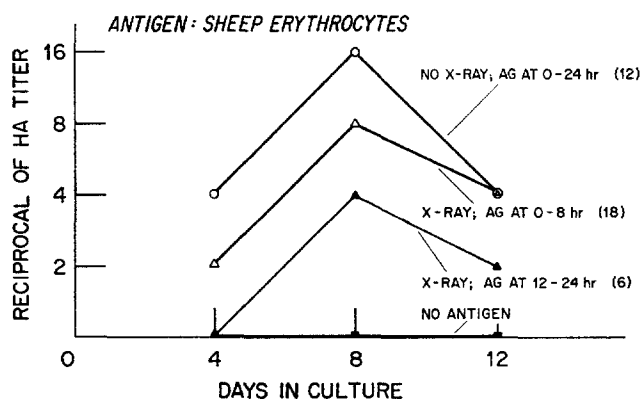


FIG. 4. Average hemagglutination (HA) titers of culture fluids removed every 4 days from tissue receiving a secondary exposure to antigen (AG = sheep erythrocytes) in vitro at varying intervals after radiation (500 R). The numbers in parentheses following the line labels indicate the number of culture tubes used in each experimental group.

resistant than the one to DT. No antibody production was observed by tissue not reexposed to antigen in vitro.

During the course of this work it was noted that lymph nodes from rabbits sensitized to SE must be used within 3-5 months in order to obtain an optimal secondary response. This was in sharp contrast to BSA- and DT-sensitized tissues from which a secondary response in vitro could be elicited even 18 months after primary stimulation. It was also noted that the secondary response, induced in vitro in tissue taken from SE-sensitized animals 2-4 wk after the primary injection, consisted mostly of 0.1 M mercaptoethanol-resistant antibody. Although the peak titers obtained with a 90-150 day interval before the in vitro challenge were not lower, the antibody produced in these cases was partially sensitive to mercaptoethanol and labile upon storage at  $-20^{\circ}\text{C}$ .

*Effect of Drugs Interfering With Cell Proliferation.*—The effect of X-irradiation was compared to that of other agents known to affect cell proliferation either

by interfering with DNA metabolism (5-bromodeoxyuridine) or by inhibiting mitosis (colchicine). Lymph node tissue from DT-sensitized rabbits was re-exposed to DT in vitro and then exposed to a concentration of 250  $\mu\text{g}/\text{ml}$  of BUDR in the medium during days 0-4 or 8-12. Fig. 5 shows that the exposure to BUDR during the first 4 days of the tissue culture period completely inhibited antibody formation, whereas the presence of BUDR in the culture medium during days 8-12 had no appreciable effect.

Colchicine added in a concentration of 0.4  $\mu\text{g}/\text{ml}$  during days 8-12 greatly inhibited antibody production (Fig. 5). There may have been a delay before colchicine completely stopped antibody production, but no further attempts

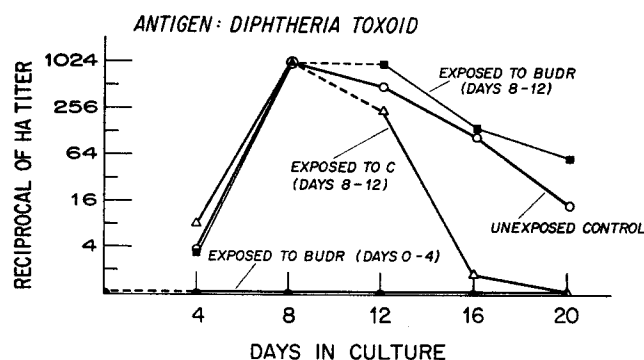


FIG. 5. Average hemagglutination (HA) titers of culture fluids removed every 4 days from tissue subjected to 5-bromodeoxyuridine (BUDR) or colchicine (C) at varying intervals after secondary exposure to DT in vitro. The broken portions of the lines indicate the periods during which the drug was present in the medium. Results of two experiments (four culture tubes per point) are shown for each line.

were made to investigate this point. Neither BUDR nor colchicine interfered with the production of acid in the culture tubes due to the metabolism of the tissue fragments. It was noted that both drugs inhibited the outgrowth of fibroblasts from tissue fragments.

#### DISCUSSION

The present results show that the sensitivity to X-irradiation of the secondary response in vitro varies with respect to the time interval between antigen exposure and irradiation. The temporal aspects of this variation are quite similar to those observed by others for the primary response in vivo. X-Irradiation inhibits the response most effectively when given before antigen. When a period of 24 hr is allowed to elapse after X-irradiation before antigen is given, the response is completely inhibited.

This similarity between the secondary response in vitro and the primary response in vivo agrees well with the conclusion reached by Makinodan et al.

(10), that there is no difference in sensitivity to X-irradiation between the cells reacting during a primary and a secondary response, but that there is a marked quantitative difference in the numbers of cells responding. Since only a limited amount of tissue is present in the culture tubes, the secondary response in vitro is quantitatively different from the one in vivo. This is evident from the relatively slow development of the response in vitro. Under certain conditions a high level of circulating antibody is known to inhibit antibody formation in vivo (17). This effect may complicate the study of the secondary response in vivo, since a range of different numbers of responding cells may give similar plateau levels of antibody in the serum. Because the culture fluids are changed every 3-4 days, this effect is minimized with the in vitro response. These circumstances therefore render the secondary response in vitro more amenable to the study of irradiation effects than the in vivo response.

Studies dealing with the effect of X-irradiation on exteriorized spleen have suggested that circulating cells settle in the irradiated spleen and give a blast cell and immature plasma cell proliferation in the periarteriolar sheaths and surrounding red pulp (18). Sussdorf and Draper (19) have shown that upon whole-body X-irradiation combined with shielding of an individual lymphoid organ such as the appendix, cells from the shielded organ go to the spleen. Porter reported that X-irradiation given 58 days after a primary injection permanently diminishes the ability of the rabbit to respond to a secondary antigenic stimulus (20). These lines of research suggest that the cell type reacting to a primary or secondary stimulus with antigen may be the circulating small lymphocyte. In fact, lymphocytes may undergo a similar blast cell transformation during the initial phase of the antibody response as the one which was shown by Gowans (21) to occur during the GVH reaction in vivo, and by Nowell (22) during the reexposure of circulating leukocytes to antigen in vitro.

Lymphocytes are known to be more sensitive to X-irradiation than most other cell types, including lymphoid blast cells (23, 24). This has been related to the effect of X-rays on oxidative phosphorylation (25). If the effect of irradiation on lymphocytes is indeed responsible for the suppression of antibody formation, it has to be assumed that there is a critical period after irradiation in which the lymphocytes can be protected from the lethal effects by exposure to antigen. The present results demonstrating a relative lack of responsiveness of tissue reexposed to antigen at 12-24 hr, as opposed to tissue reexposed 0-6 hr after irradiation, clearly show that the protective effect of antigen is through its action on cells located in the isolated lymphoid tissue. It seems possible that during the transformation of lymphocyte to blast cell certain enzymes are acquired and metabolic changes occur which prevent the cells from dying. In the present study this reversal of the radiation induced damage could take place only as long as antigen was given within 12 hr after irradiation.



Although the phagocytic and degradative properties of macrophages are not sensitive to radiation (26, 27), a recent report nevertheless suggests that a major effect of X-ray on antibody formation is through its effect on macrophages rather than on lymphocytes (28). These latter studies were performed with a bacterial antigen (*Shigella*) and still remain to be confirmed and repeated with protein antigens. The difference in effectiveness of X-ray on tissue receiving antigen immediately as compared to tissue receiving antigen 12–24 hr later, could possibly be explained on the basis of an inhibitory effect of irradiation on a unique antigen-processing function of macrophages (29, 30). If this were the case, however, it is clear that the irradiated macrophages must lose this special property over a period of 12–24 hr after irradiation. The depressing effect of X-irradiation on the secondary response in vivo, is probably not mediated by an inhibition of macrophages. Porter has shown that this effect is permanent and can still be shown at a time after irradiation when the animals are again capable of a normal primary response (31).

When irradiation is applied to cells which have already transformed into blast cells, the degree of effectiveness of irradiation appears governed by its effect on cell proliferation. Both X-irradiation and BUDR are only effective in inhibiting to varying degrees the secondary response in vitro when applied during the initial period after exposure to antigen. Neither agent is able to affect antibody formation to a significant degree when applied after the peak of the response (X-irradiation, day 6; BUDR, days 8–12). It seems that cell proliferation is no longer of crucial importance for the in vitro secondary response at this time (12). At present it remains difficult to understand the mechanism whereby colchicine is still able to block the antibody synthesis when added after the peak of the response, since most of the known effects of colchicine are on cell proliferation. A similar observation has been reported by Dutton and Pearce (32).

Unchallenged lymph node tissue from animals sensitized with BSA, even when taken as long as 18 months after a single immunizing dose, produced a spontaneous antibody response in vitro. Occasionally this response was equal to the one observed with challenged tissue. To a lesser degree this was also observed by Michaelides et al. (11). It seems unlikely that a nonspecific stimulation due to the tissue culture conditions is responsible for this phenomenon since DT-sensitized tissue never exhibited such a response. It is also unlikely that a comparable level of antibody synthesis was still occurring in the animals since antibody could not be detected in their sera or in the initial medium changes of the cultures. The sensitivity to X-irradiation of this response was similar to that of tissue which was reexposed to antigen at the onset of the culture period. It seems possible that small amounts of BSA remain in the tissue for extended periods (33), and become redistributed and available to act as challenging antigen during the preparation of the tissue cultures.

## SUMMARY

The present studies have shown that the influence of X-irradiation on the secondary antibody response in vitro is remarkably similar to its effect on the primary response in vivo.

When sensitized tissue was first irradiated and then reexposed to antigen, the duration of the interval between irradiation and antigen addition determined the degree of inhibition of the secondary response obtained. A delay of 12 hr resulted in stronger inhibition than a delay of 6 hr, and an interval of 24 hr before reexposure to antigen caused complete suppression of antibody production to diphtheria toxoid and almost complete suppression when sheep RBC were used as the antigen.

Induction of the secondary response in rabbit lymph node tissue in vitro followed by exposure to X-irradiation, revealed that immediate exposure to irradiation after antigen produced stronger inhibition of the subsequent response than irradiation on days 2-3. Irradiation on day 6 had no detectable effect. The effectiveness of the early radiation is probably due to prevention of the proliferation of the antibody-forming cells. BUDR was found to be effective at similar time periods as X-irradiation, whereas colchicine could still stop antibody formation when added late during the secondary response in vitro.

It was noted that lymph nodes from some BSA-sensitized rabbits as late as 18 months after sensitization gave a response indistinguishable from a typical secondary response, even when not reexposed to antigen.

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