Inhibition of Secondary Immune Responses *in Vivo* by Immunoregulatory Alpha Globulin (IRA)[1] (35982)

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(Introduced by R. H. Egdahl)

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Several investigators, including Kamrin (1), Mowbray (2), Carpenter (3), Riggio *et al.* (4), Milton (5), and the present authors (6) have demonstrated that alpha globulin-rich plasma protein fractions will suppress the primary immune response to a variety of antigens. An alpha globulin fraction which we have called immunoregulatory alpha globulin (IRA) suppressed antigen-induced proliferation of lymphocytes from specifically sensitized individuals (7), a finding which suggested that these alpha-globulin preparations might also suppress secondary immune responses. Using a standard hemolytic plaque assay the present studies explore the effect of IRA on the formation of hemolysin-producing cells in the secondary as well as the primary immune response of mice to sheep red cells (SRC).

Materials and Methods. Animals. Adult C57BL/6J mice weighing approximately 20 g (obtained from Jackson Memorial Laboratory, Bar Harbor, ME) were maintained on a standard laboratory diet and water *ad libitum.*

IRA preparations. The IRA used in the present studies was prepared from human plasma proteins by cold ethanol fractionation. Two kilograms of Cohn fraction IV paste (Biologic Laboratories of the Massachusetts Department of Public Health, Jamaica Plain, MA) were suspended in 10.4 liters of 36% ethanol, buffered to pH 5.2 and ionic strength 0.18 with sodium acetate and acetic acid. Temperature was constant at $-5°$. The ethanol concentration was then lowered by the addition of 480 ml of acetate buffer and 1.4 liters of cold distilled water. The precipitate, which formed overnight, was removed by centrifugation, dialyzed against distilled water, and lyophilized. Prior to injection into mice all IRA preparations were brought to pH 7.4 by dialysis against phosphate buffered saline or by reconstitution of lyophilized material with Hanks' balanced salt solution and were then sterilized by passage through 0.45 μ Millipore filters (Millipore Filter Corporation, Bedford, MA). Treated mice received IRA protein in a 0.5 ml volume injected in a tail vein.

Immunizations. Mice were immunized and challenged with tail vein injections of 0.25 ml of a 25% suspension of SRC obtained fresh weekly from Colorado Serum Company. When required, the cells were washed twice in saline and finally resuspended in saline to an appropriate volume for injection.

Plaque assays. Direct hemolytic plaques of both the primary and secondary response were measured by a minor modification (8) of the Jerne hemolytic plaque assay. Indirect plaques of the secondary response were assayed according to the technique of Dresser and Wortis (9) by adding rabbit antiserum to mouse IgG (Microbiological Associates) to the plates after the first incubation and before the addition of complement. Plaque numbers were recorded as plaques per 10^6 spleen cells. Indirect plaques were the number of plaques obtained by subtracting the number of direct plaques from the number of total plaques formed by the addition of rabbit antimouse IgG serum. Plaque assays were performed in triplicate, or rarely, in dupli-

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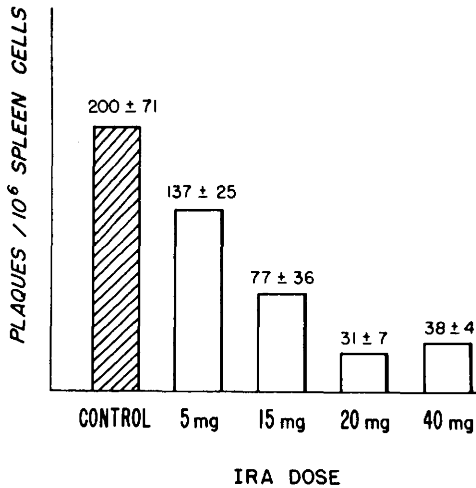

FIG. 1. Effect of graded doses of IRA on primary direct plaque-forming cell response. Assays for hemolytic plaque-forming cells were performed 4 days after SRC injection. IRA was administered 20 hr prior to SRC. Each value is the mean ± one standard deviation of triplicate assays of three or four mice.

cate on three or four mice from each group in each experiment. Results are expressed as the mean of the total assays in each group.

Results. Graded doses of IRA preparation, ranging from 5 to 40 mg, were administered 16 to 20 hr prior to SRC injection. Control animals were either untreated or received equivalent amounts of bovine serum albumin (BSA) (Mann Research Laboratories, Orangeburg, NY) at the time their counterparts received IRA. All animals were sacrificed 4 days after the SRC injection and plaque assays were performed for primary hemolytic plaque-forming cells. As shown in Fig. 1, there was a progressive inhibition of plaque-forming cells with increasing doses of IRA until a dose of 20 mg was reached. Increasing the dose further produced no further inhibition of plaque formation. Equivalent quantities of BSA injected at times corresponding to the time of injection of the IRA fraction gave no suppression. Equivalent quantities of whole human serum or of other Cohn fractions of human plasma were similarly tested and found not to be suppressive.

In vivo, *duration of IRA effect.* To determine the *in vivo* duration of IRA activity, mice were injected with IRA then SRC 1, 2, 3, 4, 6, 8, and 9 days later. Direct plaque assays were performed 4 days after the SRC injection. The number of hemolytic plaques per 10^6 spleen cells from IRA-treated animals are plotted as a percentage of hemolytic plaques per 10^6 spleen cells from control animals in Fig. 2. IRA exerted its greatest effect when it was administered 1 day prior to the SRC injection. When the interval between the IRA and SRC injection was lengthened, the immunosuppressive effect gradually diminished. If SRC were given 4 days after the IRA injection, the inhibition of plaque formation was decreased to approximately 40%; by 7 days, there was no longer any inhibitory effect. At 9 days, there was no reappearance of inhibitory effect.

Magnitude of the secondary plaque response. In studying the secondary immune response, animals were immunized with SRC and 9 days later were challenged with an identical dose of SRC. To determine the time of maximum secondary hemolytic plaque-forming response, assays for direct plaques were performed on days 2, 3, 4, and 5 after the challenge, or second injection of SRC (Fig. 3). In the control group, which con-

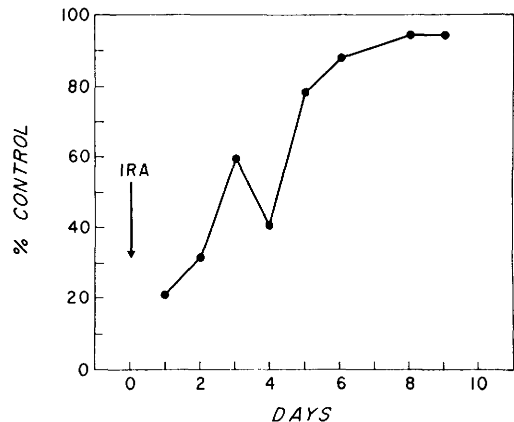

FIG. 2. *In vivo* duration of IRA effect. The mean number of hemolytic plaques per 10^6 spleen cells from three IRA-treated animals is plotted as a percentage of hemolytic plagues per 10^6 spleen cells from three control animals. IRA was administered to all treated animals on day 0. The time scale indicates the interval between IRA administration and performance of the assay.

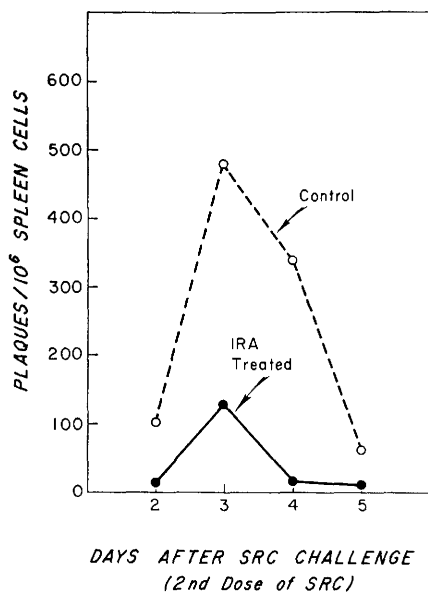

FIG. 3. Magnitude of the secondary plaque-forming response. Animals were immunized with SRC and 9 days later were challenged with an identical dose of SRC. Treated animals received IRA intravenously 16 to 20 hr prior to both immunization and challenge. Each point represents the mean of six animals.

sisted of animals immunized with SRC and challenged with a second injection of SRC 9 days later, the peak plaque-forming response occurred on day 3 after the challenge. In the experimental group, when animals were injected with IRA 24 hr prior to both immunizing and challenge dose of SRC, the peak plaque-forming response also occurred 3 days after the challenge dose. The peak response of the IRA-treated group reached a mean of 120 plaques/10^6 spleen cells as compared to the peak response in the control group which was 480 plaques/10^6 spleen cells. Because the peak direct secondary plaque response occurred on day 3 after the challenge dose of SRC in both the control and IRA-treated groups, this day was chosen for plaque assays of the secondary response in all subsequent experiments.

Inhibition of the secondary immune response by IRA administered prior to immunization. Animals injected with IRA 24 hr prior to the immunizing or first injection of SRC were challenged with a second dose of SRC 9 days after the initial SRC injection. Both direct and indirect hemolytic plaques were assayed 3 days after the SRC challenge dose. The secondary direct plaque-forming response was 66% suppressed (Fig. 4), and there was a 54% suppression of the secondary indirect plaque-forming response. Since earlier experiments had shown that the *in vivo* effect of IRA was greatly diminished by 4 days and was completely absent by 7 days after the injection of IRA, it seemed unlikely that suppression of the secondary immune response was related to persistence of IRA effect.

Suppression of the secondary immune response by IRA administered prior to challenge. Animals immunized with SRC were challenged with a second dose of SRC 9 days later. IRA was administered 24 hr prior to the challenge injection of SRC. Direct and indirect plaques were assayed 3 days after the challenge injection (Fig. 5). IRA-treated animals showed a significant suppression of the secondary direct hemolytic plaque response of 69%. However, IRA-treated animals had a mean of 66 secondary indirect plaques/10^6 spleen cells, while the control

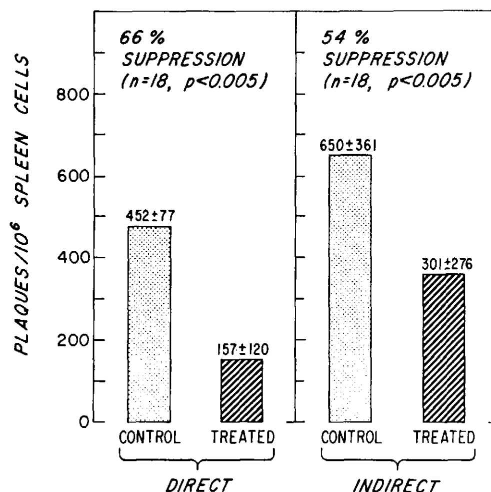

FIG. 4. Inhibition of the secondary response by IRA administered prior to immunization. IRA was administered 1 day prior to immunization with SRC. A challenge dose of SRC was administered 9 days after the initial injection of SRC. The means represent duplicate assays on nine mice in each group.

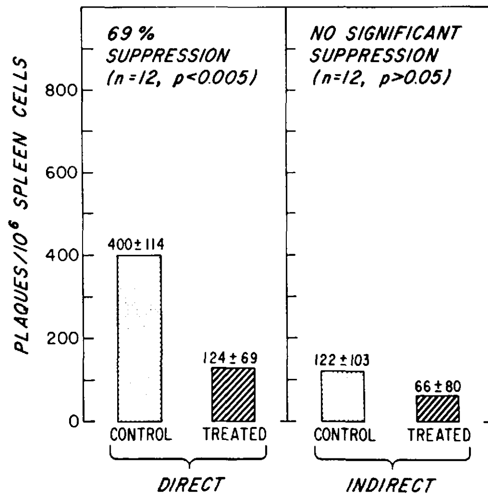

FIG. 5. Suppression of the secondary immune response by IRA administered prior to challenge. Animals immunized with SRC were challenged with a second dose of SRC 9 days later. IRA was administered 1 day prior to the challenge injection of SRC. The means represent duplicate assays on six mice in each group.

group had 122 plaques/10^6 spleen cells. This difference was not significant.

Inhibition of the secondary immune response by IRA administered prior to both immunization and challenge. Animals immunized with SRC were challenged 9 days later with a second injection of SRC. IRA was administered 24 hr prior to both the immunizing injection of SRC and the challenge injection of SRC. Only direct hemolytic plaques were assayed. In the IRA-treated group, there was 78% suppression of the secondary direct hemolytic plaque response (Fig. 6). This degree of suppression of the secondary response was equivalent to the degree of suppression of the primary response by IRA in prior experiments; however, administration of IRA prior to both primary and challenge injections of SRC did not convert the secondary response to a primary response as indicated by the fact that on day 3 there was still a mean of 84 plaques/10^6 spleen cells in the IRA-treated groups; whereas earlier experiments had shown that on day 3 following a primary immunization with SRC there were 0 to 10 plaques/10^6 spleen cells in both control and IRA-treated animals.

Discussion. These results demonstrate that alpha globulin-rich fractions isolated from normal human plasma can suppress both the primary and secondary immune response in mice to SRC as measured by hemolytic plaque assay. In the primary response to SRC, prior IRA administration yielded 80% suppression of direct hemolytic plaque formation. IRA was also effective in suppressing the secondary direct plaque-forming response whether it was administered prior to the immunizing, or first, injection of SRC, or prior to the challenge, or second, injection of SRC. This demonstrates that IRA administered at the time of initial antigen exposure will not only suppress the primary immune response to that antigen but will also suppress the secondary immune response to a subsequent challenge by the same antigen. In this series of experiments the challenging, or second, dose of SRC was administered at a time when all the immunosuppressive activity of the initial IRA injection had dissipated; yet both the direct and indirect secondary plaque-forming responses were suppressed. It is possible that IRA administered at the time of initial antigen stimulation prevented the development of memory cells, however,

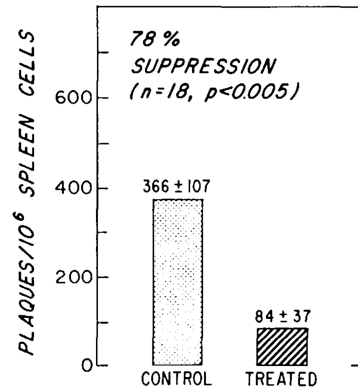

FIG. 6. Inhibition of the secondary immune response by IRA administered prior to both immunization and challenge. Animals immunized with SRC were challenged 9 days later with a second injection of SRC. IRA was administered 1 day prior to both injections of SRC. Only direct plaques were assayed. The means represent duplicate assays on nine mice in each group.

the secondary response obtained after challenge with antigen was significantly less than the normal primary response and suggests that the initial IRA treatment administered 1 day prior to the immunizing injection of antigen conferred partial tolerance to that antigen.

It is unlikely that this immunosuppression is due to antigenic competition as there was no suppression with equivalent quantities of human serum or other Cohn plasma protein fractions. Moreover, we have previously demonstrated by *in vitro* experiments that human IRA will suppress human lymphocytes (7), and guinea pig Cohn fraction IV will suppress guinea pig lymphocytes (10). Toxicity is not the cause of this effect. IRA injections have been well tolerated, there being no change in weight, hematological profile, or in the structure of the lymphoid organs (11).

The fact that IRA was effective in suppressing the secondary direct plaque-forming response when it was administered prior to the challenge, or second, injection of sheep red cells indicates that IRA will suppress the secondary immune response even in an animal with an already developed state of immunity. These results differ from those reported by Mowbray (12) who found that a similar alpha globulin fraction extracted from bovine serum inhibited the first set rejection response but had little effect on the second set rejection of skin allografts in rats and rabbits. This difference in results may be attributed to the different origins of the alpha globulin fractions or to the difference in assays used to test for suppression.

Summary. These studies demonstrate that IRA will suppress both primary and secondary immune responses as measured by a hemolytic plaque assay. Suppression of the secondary response occurs whether IRA is administered prior to the immunizing injection of antigen or prior to the challenge injection of antigen. The fact that IRA administered at the time of initial antigen exposure was able to inhibit the response to a second, or challenge, dose of that antigen; and that the secondary response was significantly less than a normal primary response suggests that IRA administered at the time of initial antigen exposure confers partial tolerance to that antigen.

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Vol. **136**, No. 4 (1971), "*In vitro* Demonstration of Potassium Excretion by the Toad Bladder," by F. T. Kallus, J. C. Vanatta, W. H. Burke, and E. A. Hetherington, pp. 1245–1248:

Page 1247, first column, line 26, ". . . the S $\rightarrow$ M flux averaged 2.69 . . ." should read: ". . . the S $\rightarrow$ M flux averaged 3.69 . . ."

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