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The Role of Macrophages in the Induction of Antibody in X-Irradiated Animals

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Summary. A study was carried out on the function of macrophages in inducing antibody production to Shigella antigen, and on the effect of X-irradiation on the 'immunogenic' function of macrophages. Peritoneal macrophages, which had been incubated with Shigella and then injected into mice exposed to 550 r, triggered the formation of agglutinating antibody in animals which did not respond to the injection of the antigen alone. The antibody formed was not produced by 'contaminating' lymphocytes of the peritoneal exudate, since: (a) lymph node cells at doses higher than those of the macrophage inocula did not produce antibody when treated and injected under similar conditions, and (b) lymphocyte-free macrophage populations, obtained by culturing *in vitro* cells of peritoneal exudates, triggered the production of antibody when injected in to X-irradiated recipients after interaction with the antigen. Macrophages from irradiated donors incubated with Shigella were incapable of inducing antibody formation in X-irradiated mice. Animals exposed to higher doses of irradiation (900 r) did not produce antibody following injection of macrophage–antigen complexes. It was, therefore, concluded that macrophages from normal animals elicited the production of antibody by the lymphoid cells of the irradiated recipients.

INTRODUCTION

The suppression of immunological reactivity in animals following total body X-irradiation has been generally attributed to the destruction of the lymphoid tissues. Although it has been claimed that macrophages play a decisive role in triggering the immune response (Fishman, 1961), their 'immunogenic' capacity following irradiation has not been studied. On the basis of histological evidence, RES (reticulo-endothelial system) macrophages appeared to be radiation-resistant (Brecher, Endicott and Broner, 1948; Bloom, 1948); most studies have seemed to indicate that the clearance of colloidal particles by the RES is unaffected by total body irradiation (Barrow, Tullis and Chambers, 1951; Wish, Furth, Sheppard and Storey, 1952; Gabrieli and Auskaps, 1953; di Luzio, 1955; Benacerraf, Biozzi, Halpern and Stiffel, 1957; Benacerraf, Kivy-Rosenberg, Sebestyen and Zweifach, 1959). Similar observations have been made with regard to bacteria; here, however, it appears that although the initial clearance of bacteria is unaffected by X-rays, macrophages of irradiated animals are unable to kill or retain bacteria (Gordon, Cooper and Miller, 1955). Peritoneal macrophages of irradiated rabbits showed a reduced capacity to digest chicken red blood cells, as compared to those of non-irradiated donors (Donaldson, Marcus, Gyi and Perkins, 1956). Since macrophage–antigen interaction may be an essential step in the induction of antibody production, the impaired

capacity of macrophages of X-irradiated animals to degrade antigenic material could at least partly account for the impaired immunological reactivity of animals exposed to sub-lethal doses of X-rays.

In the present study, experiments were carried out on the capacity of macrophages from normal, as compared to those from X-irradiated mice, to elicit antibody production in irradiated mice, when injected after an *in vitro* interaction with Shigella antigen. The results were expected to indicate whether the processing of antigen by macrophages is a determining step in triggering antibody production.

MATERIALS AND METHODS

Animals

Female mice of C57BL/6 strain, 10 weeks old, were used both as donors and recipients in all the experiments. The sera of these mice were tested for agglutinating 'natural' antibody to Shigella. No measurable traces of antibody could be found.

X-irradiation

Mice were exposed in lucite containers to total body X-irradiation of 550 r (unless otherwise stated), using a General Electric Maximar III X-Ray machine (250 kV, 15 mA). They were irradiated at a target distance of 50 cm (dose rate 65 r/min) with 0·5 mm Cu and 1 mm Al filters.

Peritoneal macrophages

Macrophages were obtained from the peritoneal exudate by a modification of Bang's method (Gallily, Warwick and Bang, 1964). Each mouse was injected intraperitoneally with 3 ml thioglycollate medium (Difco Laboratories, Detroit, Michigan, U.S.A.). Four days later, the peritoneal cells were collected by washing out the peritoneum with 5 ml phosphate-buffered saline containing penicillin, streptomycin and heparin (100 units, 50 μg and 5 u.s.p. units/ml, respectively). The cells were counted with a haemocytometer and after centrifugation (1000 rev/min for 8 minutes) were suspended in Hanks's salt solution. Incubation of these cells with Shigella was then carried out in Erlenmeyer flasks containing $7{\cdot}5 \times 10^6$ cells/ml of 0·005 per cent Shigella suspension in Hanks's solution, on a shaking tray for 1 hour at 37°. After incubation, the cells were centrifuged and washed in approximately 40 volumes of Hanks's salt solution. They were then resuspended in Hanks's solution at a concentration of 30×10^6 cells/ml, and injected intraperitoneally at doses of 15×10^6 cells per recipient 2 days after exposure of the mice to X-rays.

Macrophages in tissue culture

The macrophages were taken from the peritoneum as described previously. After sedimentation, the packed cells were resuspended in a tissue culture medium (horse or foetal calf serum 35 per cent, chick embryo extract 5 per cent and Hanks's salt solution). The cells were then transferred into 60 mm diameter Petri dishes (6×10^6 cells/dish) which had been overlayed with reconstituted rat tail collagen substrate, prepared according to the method of Ehrmann and Gey (1956). After 48 hours in culture about 98 per cent of the cells that adhered to the bottom of the dishes were macrophages. These macrophages were then washed twice with Hanks's solution and Shigella suspension was added (final concentration 0·005 per cent) to the cell-containing medium which consisted of foetal calf serum

(10 per cent) and Hanks's solution. The Petri dishes were incubated for 1 hour at 37°. The cultures were then washed with Hanks's solution and collagenase (0·025–0·050 per cent) was applied to remove the cells. Incubation with the enzyme was carried out for 15 minutes in a shaking tray at 37°. The released cells were washed, resuspended, and after counting were injected intraperitoneally into the X-irradiated mice.

Lymphocytes

The cervical, submaxillary, axillary, brachial, inguinal and mesenteric nodes were excised from normal mice. The nodes were cut in Tyrode's solution, filtered through a stainless steel filter (pore size of $0{\cdot}6 \times 0{\cdot}6$ mm), and washed twice. After centrifugation, the cells were suspended with the antigen in Hanks's solution at a concentration of $7{\cdot}5 \times 10^6$ cells/ml of 0·005 per cent Shigella suspension and incubated for 1 hour at 37° on a shaking tray. The cells were then washed, resuspended in Hanks's solution, and injected intraperitoneally into X-irradiated mice.

Antigenic material

The antigen used was *Shigella paradysenteriae.* The stock of bacteria was obtained by courtesy of Dr T. N. Harris, and was prepared according to his description (Harris, Harris and Farber, 1954). A 10 per cent suspension (v/v) of the alcohol-killed bacteria was kept at 4° as a standard stock.

Anti-Shigella agglutinins

Agglutinins were measured after serial two-fold dilutions of 0·1 ml volumes of mouse sera and a subsequent addition of 0·5 ml of 0·02 per cent suspension of alcohol-treated Shigella per tube. After shaking and 1 hour of incubation at 37°, the tubes were stored at 4° for 48 hours, then read for agglutination by the pattern of sediment on the bottom of the tubes, and also by shaking them under the agglutinoscope.

Cytologic examination

Smears of cell suspension before incubation were prepared and stained by Giemsa or May–Grünwald–Giemsa. Differential counts were made of 400–600 cells in each test.

RESULTS

The first set of experiments aimed at testing whether macrophages from normal donors could, after interaction *in vitro* with Shigella antigen, trigger an immune response in mice which had been exposed to sublethal doses of total body irradiation. Macrophages from the peritoneal exudates of normal C57BL mice were incubated with Shigella, then injected at doses of 15×10^6 cells per animal into mice which had been exposed 2 days previously to a sublethal dose of 550 r total body irradiation. One control group of irradiated recipients was inoculated with macrophages alone (without pre-incubation with antigen), another with Shigella alone. The animals were bled and titrated 5 and 8 days following treatment. The results of a number of such experiments are pooled and summarized in Table 1. They indicate that macrophages from normal animals after interaction with the antigen elicited the formation of antibody in the X-irradiated mice, which showed a $\log_2$ titre of 4·0 after 5 days, rising to 7·5 when measured after 8 days. The agglutinating titre of the control groups did not rise above 1·6 (Table 1).

TABLE 1

ANTIBODY PRODUCTION BY IRRADIATED C57BL MICE FOLLOWING INOCULATION OF MACROPHAGES INCUBATED *in vitro* WITH SHIGELLA ANTIGEN

Treatment of irradiated recipients (per mouse)	No. of experiments	Agglutinin titre: 5 days			Agglutinin titre: 8 days		
		No. of tested mice	$\log_2$ of titre (mean)	S.E.*	No. of tested mice	$\log_2$ of titre (mean)	S.E.*
15×10^6 macrophages incubated with Shigella	6	36	4·0†	0·10	54	7·5†	0·37
15×10^6 macrophages	4	19	0·1	0·07	30	0·8	0·29
Shigella (0·1 ml of 0·1 per cent suspension)	6	27	0·7	0·30	40	1·6	0·30

* Standard error of the mean.
† The difference between this value and that of each of the two control experiments was checked according to the rank sum test and found significant at a level exceeding 99 per cent.

The reactivation of the immune response in the irradiated recipients as a function of the inoculated cell dose was investigated. Mice exposed to 550 r were inoculated 2 days later with different doses of macrophages which had been incubated with Shigella antigen. The results are recorded in Table 2 and Fig. 1. In view of the clear and sufficiently high immune response obtained in animals treated with 15×10^6 cells this dose of macrophages was chosen as the standard one in all the following experiments.

TABLE 2

ANTIBODY PRODUCTION BY IRRADIATED C57BL MICE AS A FUNCTION OF MACROPHAGE DOSE

Treatment of irradiated recipients (per mouse)	No. of experiments	Agglutinin titre: 5 days			Agglutinin titre: 8 days		
		No. of tested mice	$\log_2$ of titre (mean)	S.E.*	No. of tested mice	$\log_2$ of titre (mean)	S.E.*
$2·5\times10^6$ macrophages incubated with Shigella	2	10	0·5[(a)]	0·28	15	3·4[(d)]†	0·44
5×10^6 macrophages incubated with Shigella	2	7	1·4	0·49	7	2·1	0·83
10×10^6 macrophages incubated with Shigella	2	8	1·5	0·39	15	4·2	0·41
15×10^6 macrophages incubated with Shigella	2	8	3·1[(b)]	0·35	16	7·1[(e)]	0·88
80×10^6 macrophages incubated with Shigella	3	10	8·0[(c)]	0·44	11	13·0[(f)]	0·36
80×10^6 macrophages	2	5	0	–	8	0·8	0·42
Shigella (0·1 ml of 0·1 per cent suspension)	3	13	0·6	0·28	20	1·9	0·18

* Standard error of the mean.
† The differences between the values of (a) and (b), and (b) and (c), as well as those between (d) and (e), and (e) and (f) were checked according to the rank sum test and found significant at a level exceeding 99 per cent.

The macrophage cell population of the peritoneal exudate used in our experiments was found to be contaminated with about 10 per cent lymphocytes (Table 3). Therefore, the formation of agglutinins to Shigella in the irradiated recipients obtained in the previous experiments might be attributable to the effect of the lymphoid components of the peritoneal exudates. To test whether in fact the inoculated lymphocytes rather than the macrophages

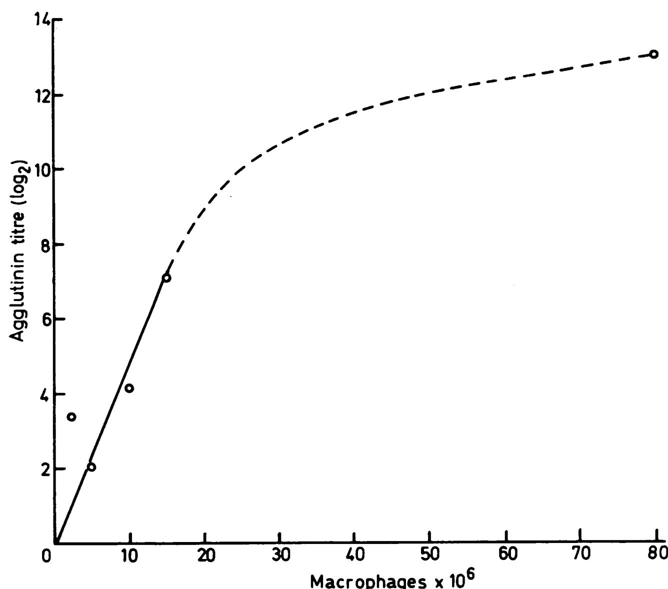

FIG. 1. Antibody production by irradiated C57BL mice as a function of macrophage dose.

TABLE 3

DIFFERENTIAL COUNT OF PERITONEAL CELLS BEFORE AND AFTER INCUBATION WITH SHIGELLA

Treatment	No. of cells counted	Macrophages (per cent)	Granulocytes (per cent)	Lymphocytes (per cent)
Cells	4400	83	7	10
Cells + Shigella	1400	87	5	8

were the cells that determined the formation of agglutinins to Shigella in the irradiated recipients, the following experiments were carried out. Lymphocytes obtained from the lymph nodes of normal mice and incubated with Shigella under conditions similar to those of the macrophage–Shigella incubation, were injected at various cell doses into C57BL mice that had been exposed 2 days previously to 550 r. The results (Table 4) show clearly that lymphocytes, even at doses greater by a factor of seven than those injected together with the macrophage population, elicited only a very low titre of antibody—less by a factor of 10 than the titre obtained following the inoculation of macrophages. It appeared, therefore, that the macrophages rather than the lymphocytes had triggered the formation of antibodies in the irradiated mice.

It could be postulated that, while the macrophages 'process' the antigen, it is the lymphocytes within the inoculated macrophage population rather than those of the recipients that form the antibodies in response to the 'macrophage–antigen' message. Were this so, a result similar to the previous one should be obtained if macrophages that had interacted with antigen were injected into animals exposed to doses higher than 550 r. On the other hand, if the macrophages 'signal' the lymphocytes of the recipient animals

TABLE 4

ANTIBODY PRODUCTION BY IRRADIATED MICE FOLLOWING INOCULATION OF MACROPHAGES OR LYMPHOCYTES INCUBATED *in vitro* WITH SHIGELLA ANTIGEN

Treatment of irradiated recipients (per mouse)	No. of experiments	Agglutinin titre					
		5 days			8 days		
		No. of tested mice	$\log_2$ of titre (mean)	S.E.*	No. of tested mice	$\log_2$ of titre (mean)	S.E.*
15 × 10^6 macrophages incubated with Shigella	6	36	4[(a)]†	0·10	54	7·5[(c)]†	0·37
1·5 × 10^6 lymphocytes incubated with Shigella	2	7	0	–	11	1·8	0·54
5 × 10^6 lymphocytes incubated with Shigella	3	15	0·1	0·05	23	1·0	0·36
10 × 10^6 lymphocytes incubated with Shigella	2	7	0[(b)]	–	10	1·6[(d)]	0·60
10 × 10^6 lymphocytes	2	8	0	–	11	1·5	0·51
Shigella (0·1 ml of 0·1 per cent suspension)	3	11	1·4	0·57	11	1·6	0·57

* Standard error of the mean.
† The difference between the values of (a) and (b) as well as the difference between (c) and (d) were checked according to the rank sum test and found significant at a level exceeding 99 per cent.

that survived irradiation to produce antibodies, the higher the dose of irradiation the lower should be the immune response of the host, since depletion of lymphocytes would leave less target cells for the macrophage 'signal'. To test this, mice exposed to 550, 750 and 900 r respectively were inoculated with macrophages following incubation with the antigen. The results (Table 5) show that animals exposed to 550 r responded with a titre

TABLE 5

THE PRODUCTION OF ANTIBODY BY ANIMALS EXPOSED TO VARIOUS DOSES OF X-RAYS AND INOCULATED WITH MACROPHAGES

Treatment of recipients		No. of experiments	Agglutinin titre					
			5 days			7 or 8 days		
Irradiation (r)	Inoculation (per mouse)		No. of tested mice	$\log_2$ of titre (mean)	S.E.*	No. of tested mice	$\log_2$ of titre (mean)	S.E.*
550	15 × 10^6 macrophages incubated with Shigella	6	36	4·0[(a)]†	0·1	54	7·5[(c)]†	0·37
750	15 × 10^6 macrophages incubated with Shigella	2	16	2·6	0·38	15	3·2	0·75
900‡	15 × 10^6 macrophages incubated with Shigella	1	8	0·5[(b)]	0·1	10	1·0[(d)]	0·41
550	Shigella (0·1 ml of 0·1 per cent suspension)	6	27	0·7	0·3	40	1·6	0·3
750	Shigella (0·1 ml of 0·1 per cent suspension)	2	14	0	–	16	0	–
900‡	Shigella (0·1 ml of 0·1 per cent suspension)	1	8	0	–	5	0	–

* Standard error of the mean.
† The difference between the values (a) and (b) as well as the difference between (c) and (d) were checked according to the rank sum test and found significant at a level exceeding 99 per cent.
‡ Animals of these groups were protected by 10^6 bone marrow cells.

of 7·5, as compared to 3·5 for mice exposed to 750 r, and 1·0 for mice exposed to 900 r. It is therefore suggested that normal macrophages, following interaction with Shigella, 'instruct' lymphoid elements of sublethally irradiated mice to produce antibodies.

To eliminate further participation in the immune reaction of the lymphoid contaminants of the macrophage population, attempts were made to culture the macrophages *in vitro* and then carry out the antigen interaction. Following seeding of the peritoneal exudates in tissue culture, the macrophages adhere to the glass, whereas the lymphocytes and granulocytes remain in the tissue culture fluid. After culturing the cells for 48 hours, all attempts to remove the macrophages from the glass, either by trypsin or by a 'rubber policeman' resulted in the destruction of most of the cells. Attempts to culture the macrophages on collagen, in order to permit their removal by collagenase were, however, successful. Differential counts of the cells after 48 hours showed that 98 per cent were of the macrophage type (Table 6), while no more than 1 per cent of lymphoid cells were counted under these conditions. Such macrophages were incubated in culture with Shigella antigen, then injected into X-irradiated mice (550 r) which were subsequently tested for antibody production 5 and 8 days following cell inoculation. The results (Table 7) show that macrophages cultured *in vitro*, then interacted with Shigella, do elicit the production of agglutinin to the antigen in the irradiated recipients. Since the cultured macrophage cell

Table 6

Differential count of peritoneal cells before and after culturing *in vitro*

Origin of cells	No. of cells counted	Macrophages (per cent)	Granulocytes (per cent)	Lymphocytes (per cent)	Unidentified cells (per cent)
Peritoneal exudate after withdrawal	4400	83	7	10	–
Peritoneal exudate in culture*	2100	98	–	1	1

* Forty-eight hours after seeding.

Table 7

Antibody production following inoculation of macrophages cultured *in vitro*

Treatment of recipients (per mouse)	Agglutinin titre					
	5 days			8 days		
	No. of animals tested	$\log_2$ of titre (mean)	S.E.*	No. of animals tested	$\log_2$ of titre (mean)	S.E.*
8×10^6 macrophages on collagen incubated with Shigella	4	0	–	6	3·0	1·31
13×10^6 macrophages on collagen incubated with Shigella	7	0·6	0·23	11	4·3[(a)]†	0·72
15×10^6 macrophages on collagen incubated with Shigella	4	0	–	7	6·1[(b)]	1·55
Shigella (0·1 ml of 0·1 per cent suspension)	17	0·3	0·15	18	0·8[(c)]	0·28

* Standard error of the mean.

† The difference between the values of (a) and (c) as well as that between (b) and (c) were checked according to the rank sum test and found significant at a level between 95 and 99 per cent.

population did not contain any measurable number of lymphoid cells, it can be concluded that macrophages of normal mice can trigger cells (lymphoid?) of irradiated animals to produce antibody to Shigella. It would thus appear that macrophages of the irradiated animals cannot trigger such reactions. Accordingly it could be predicted that macrophages of X-irradiated mice after exposure to antigen would not be capable of bringing about such immune reactivation when injected into mice. To test this prediction, mice were exposed to 550 r, and after 2 days the peritoneal macrophages were withdrawn and incubated with Shigella. Controls consisted of macrophages from normal non-irradiated donors otherwise treated similarly. X-irradiated animals were then injected with one or other type of macrophage, and a third, control group, was inoculated with Shigella. The results (Table 8) clearly indicated that macrophages from normal animals elicited antibody production

TABLE 8

THE EFFECT OF MACROPHAGES FROM NORMAL AND IRRADIATED DONORS ON THE PRODUCTION OF ANTIBODIES BY IRRADIATED MICE

Treatment of irradiated recipients (per mouse)	No. of experiments	Agglutinin titre					
		5 days			8 days		
		No. of tested mice	$\log_2$ of titre (mean)	S.E.*	No. of tested mice	$\log_2$ of titre (mean)	S.E.*
15×10^6 macrophages from irradiated donors incubated with Shigella	2	22	0·4(a)†	0·17	54	0·9(c)†	0·26
15×10^6 macrophages from normal donors incubated with Shigella	2	11	3·4(b)	0·24	13	8·2(d)	0·53
Shigella (0·1 ml of 0·1 per cent suspension)	2	11	0·3	0·18	13	0·5	0·25

* Standard error of the mean.

† The difference between the values of (a) and (b) as well as the difference between (c) and (d) were checked according to the rank sum test and found significant at a level exceeding 99 per cent.

in the X-irradiated recipients, whereas those from X-irradiated donors did not. We concluded therefore that X-irradiation, at sublethal doses, impaired the immunological reactivity of the animal, not only by depleting the lymphoid cell population, but—to an even greater extent—by damaging the capacity of the macrophages to process the antigen to an 'immunogenic' state.

DISCUSSION

The experiments reported in the present study demonstrated that peritoneal macrophages from normal mice, which had been incubated *in vitro* with Shigella, triggered the production of antibody to the bacterial antigen when injected into mice which had been exposed to 550 r total body irradiation. The production of antibody by the irradiated recipients could not be attributed to the contaminating lymphocytes of the peritoneal exudates, since pure populations of macrophages obtained after *in vitro* culturing of the cells were similarly effective in inducing antibody. Neither could the production of antibody be attributed to the inoculated 'macrophages' themselves which may have undergone a 'lymphoid' transformation. This was deduced from the inverse relationship between the dose of irradiation of the recipient and the level of antibody obtained following macrophage inoculation. It was therefore concluded that cells of the irradiated recipients

produced antibody due to the triggering effect of macrophages from normal animals. Macrophages from irradiated donors were found to be incapable of eliciting antibody production after *in vitro* interaction with the antigen. It was thus demonstrated that at sublethal doses of total body X-irradiation, the immunological suppression was at least partly due to the impaired capacity of macrophages to 'process' the antigen.

It appears that this defective capacity is not due to the inability of macrophages from irradiated animals to take up the Shigella (unpublished observations). Previous studies have indicated that active phagocytosis of carbon particles or colloidal gold by RE cells of the liver, spleen and bone marrow of irradiated mice is not affected by X-irradiation (Brecher *et al.*, 1948; Wish *et al.*, 1952). In fact, the blood clearance rate of radioactive colloidal gold, ^{131}I-labelled homologous and heterologous plasma, and ^{32}P-labelled homologous and heterologous red blood cells was increased after irradiation (Wish *et al.*, 1952). The initial clearance of bacteria was as great in the irradiated animals as in the non-irradiated ones (Gordon *et al.*, 1955). However, bacteria removed from the blood stream of X-irradiated mice were not destroyed, but were released into the circulating blood. Thus, phagocytic cells of X-irradiated animals are incapable of killing and carrying out intracellular digestion of micro-organisms. Not only the lethal ability, but also the ability to encompass the intracellular degradation of killed bacteria or of chicken red blood cells is impaired in macrophages of X-irradiated animals (Donaldson and Miller, 1959). The immunogenic properties of macrophages that have interacted with bacterial antigen may depend on a specific degradation of the antigen, which may be followed by formation of a complex between the antigenic determinant and certain constitutents of the macrophages (Fishman, 1961; Askonas and Rhodes, 1965). The differences between the immunogenic properties of macrophages and granulocytes that have ingested *E. coli* may be a function of the differences in the pattern of digestion of the antigen by the two cell types (Cohn, 1962).

The demonstration that the injection of antigen alone did not elicit the production of antibody to Shigella in X-irradiated animals, while macrophage–antigen complexes did elicit this response, seems to indicate that the mere interaction between antigen and a lymphoid cell, without the functional macrophage intermediate, may not result in antibody production. It has been suggested that such interactions will induce immunological tolerance rather than antibody production (Glynn and Holborow, 1965). In our laboratory, it has been found (Nachtigal, Greenberg and Feldman, 1966) that the injection of relatively low doses of human serum albumin, as late as 4–5 weeks following exposure of rabbits to 550 r, resulted in tolerance. The lymphoid system (thymus, spleen, lymph nodes and circulating lymphocytes), had undergone nearly complete cellular regeneration by that time, yet antibody production was impaired, and immunological tolerance was conferred following antigen injection. Whether or not the impaired processing of antigen by the macrophages permitted the lymphoid cells to interact directly with the antigen, and thus acquire a state of unresponsiveness, is now under investigation.

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