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Long-Term Adjuvant Effect of Bacterial Endotoxin in Prevention and Restoration of Radiation-Caused Immunosuppression (40051)

U. H. BEHLING AND A. NOWOTNY

School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

One of the first observations of the effects of whole-body irradiation on the immune system was the resultant impaired resistance to infections evidenced by death of the animal or by quantitative measurements of the immune response to a specific antigen. Benjamin and Sluka (1) observed that exposure to x-rays resulted in the loss of antibody formation. Since that time, an overwhelming body of literature on the effects of irradiation on infections has been documented, including a large number of reviews (2–4). There is little doubt that the major cause of increased susceptibility to infection in irradiated animals exhibiting the "acute radiation syndrome" can be attributed to the loss of function of the immune system (5).

The protective effect of endotoxin against lethal irradiation was described first by Mefferd *et al.* (6) and studied in detail by others, with particular emphasis on the effect of endotoxin on hemopoiesis (7, 8). The adjuvant property of endotoxin was described first by Johnson *et al.* (9). Kind and Johnson (10) applied endotoxin simultaneously or one or two days following antigenic stimulation and observed good adjuvant effect to bovine γ globulin in rabbits. No adjuvant effect was obtained if the endotoxin was given before the antigen. Similar results were obtained by Franzl and McMaster (11), injecting mice with sheep erythrocytes following relatively high doses of endotoxin. Kind and Johnson (10) also attempted to prevent radiation-caused immunosuppression. If the antigen was given together with endotoxin, the immune response was about 50% of that elicited by antigen alone in normal animals.

Our earlier studies in this field were based on the assumption that the stimulatory effects of endotoxin on hemopoiesis and immune functions play a vital role in the protective effects of endotoxin in lethally irradiated animals. We found that the radioprotective effect was strongly time-dependent. The protection showed a biphasic profile when single low doses of endotoxic lipopolysaccharide (LPS) or of a polysaccharide-containing (PS), nontoxic fraction of LPS were administered prior to irradiation. Very recently we reported alternating immunosuppressive or immunopotentiating effects of LPS and PS, depending upon the time of their application relative to the time of immunization (12, 13). The aims in our present study were to use optimal treatment times for the adjuvant effect of endotoxin and thereby to attempt to prevent or repair the irradiation-caused immunosuppression by such endotoxin treatment.

Materials and methods. Experimental animals. All mice used were 10–14 week-old females weighing between 18–25 g. ICR mice were obtained from the Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania.

Endotoxic lipopolysaccharide (LPS). Lipid-extracted *Serratia marcescens* 08.00 cells were homogenized with 5% trichloroacetic acid (TCA). The extracted crude antigen was precipitated in cold methanol and purified by ultracentrifugation. The sediment was redissolved in deionized water, dialyzed and lyophilized.

Antigens. Sheep red blood cells (SRBC) were obtained commercially in a 25% by volume Alsever's solution from Rockland Farms, Gilbertsville, PA. The cells were washed and centrifuged three times with sterile saline, counted and adjusted to the proper cell concentration.

Radiation. The radiation source was a General Electric Maxitron 300 x-ray machine operated at 300 Kvp, 20 ma, with added filtration of 0.26 mm Cu and 1.04 mm Al, yielding a half-value layer of 1.10 mm Cu. At a source-to-target distance of 60 cm, the air exposure dose rate was between 230 and 240 r/min, as measured by a Victoreen air ionization chamber model 154 which was corrected for temperature and pressure variations.

Rosette-forming cell (RFC) assay. Our

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method involved a modified technique first described by Zaalberg (14). Mice in groups of six were injected ip with 10 μg LPS either simultaneously with SRBC or at designated time intervals, and were sacrificed by cervical dislocation 10 days following immunization. The spleens were pooled in a glass tissue grinder containing 2 ml Hank's balanced salt solution (HBSS) adjusted to pH 7.2 with $NaHCO_3$. The cell suspensions were filtered through gauze and adjusted to a concentration of 6×10^7 cells/ml HBSS. To 0.8 ml HBSS, 0.1 ml spleen cell suspension and 0.1 ml saline containing 3×10^7 SRBC were added to yield a final volume of 1 ml containing a 1:5 ratio of spleen cells to sheep erythrocytes. This suspension was vigorously agitated and immediately placed in a 4° cold room for 3 hr. Test tubes containing the samples were gently rotated at the end of incubation until the sedimented cells were uniformly resuspended before loading the hemocytometer. Only spleen cells having surface contact with a minimum of five red cells were counted as rosettes. Each sample was done in triplicate and analyzed in a blind manner.

Plaque-forming cell (PFS) assay. A modified technique of the hemolytic plaque assay first described by Jerne and Nordin (15) was used. Immunization and LPS treatment were identical to that used for the RFC assay. Again mice in groups of six were used. Spleens were obtained 4 days following immunization and pooled spleen cell suspensions were adjusted to three concentrations (by volume: 1:10, 1:100, 1:500) for plating. For an accurate determination of cell concentration, samples of these cell suspensions were stained and counted by means of a hemocytometer. Two milliliter aliquots of a 1% Noble agar solution containing (a) 0.1 ml of 1% DEAE dextran, (b) 0.1 ml of a 20% SRBC suspension, and (c) 0.1 ml of any of the various spleen cell concentrates were evenly layered on plates containing 10 ml 1.4% solidified but prewarmed Noble agar. The plates were incubated for 60 min at 37° before 2.5 ml guinea pig complement diluted with 1:10 Veronal buffer was added. The plates were incubated for an additional 30 min. At this time the complement was decanted and the plates were ready to be analyzed. Plates for each of the three concentrations of spleen cells were done in triplicate and analyzed in a blind manner.

Prevention and restoration of radiation-caused immune suppression. The time intervals between LPS injection, 400 r whole-body irradiation, and immunization with SRBC were varied in these experiments.

In the RFC immune response system, the irradiation preceded immunization by 4 days in all experiments. The time of 10 μg ip LPS treatment preceding immunization was varied from -19 days to 0 day. At day 0, the LPS and SRBC were given simultaneously ip, but by two separate injections. The immune response of the animals was measured always 10 days after immunization with SRBC, using the RFC assay.

In the PFC assay, radiation preceded immunization by either 2, 5 or 12 days. The LPS pretreatment was either 2 or 10 days before irradiation. The selection of these two time intervals was a consequence of earlier observations made in our laboratories (16), according to which the optimal time intervals for LPS-induced radiation protection peaked at these two time points. The immune response of the animals was determined by the Jerne plaque assay, always 4 days after immunization.

Whether endotoxic LPS treatment given simultaneously with SRBC immunization can restore damage caused by radiation was determined by giving LPS and SRBC 3 days after total body irradiation with 400 r.

Results. Effect of LPS on the rosette-forming cell immune response to SRBC in normal and irradiated animals. Groups of normal ICR mice (six in each group) were treated with a single dose of 10 μg LPS ip at various time intervals prior to immunization with 1×10^8 SRBC. The span of treatment extended from -19 to 0 days. The animals exhibited an enhanced RFC response which was biphasically time-dependent (Fig. 1) and closely paralleled the time-dependent survival of LPS-pretreated, lethally irradiated mice in our previous investigation (16). At shorter time intervals between LPS treatment and immunization, moderate adjuvancy could be seen. If LPS was given on days -7 or -8, suppression became evident. When LPS treatment preceded immunization by 13 days, a period of pronounced adjuvancy was observed.

In order to study the effect of LPS on the RFC response in irradiated mice, a rather high yet sublethal exposure dose of radiation (400 r) was selected. Mice in groups of six were irradiated 5 days before immunization and treated with LPS (10 μg) at identical time intervals as before (Fig. 2). Good enhancement in the number of RFC's relative to irradiated controls was most evident when LPS treatment preceded irradiation by 8–11 days (13–16 days before immunization). A biphasic time-dependent relationship was apparent again. Comparison of the RFC response of the irradiated control group with the non-irradiated controls of the previous experiments showed nearly 80% immunosuppression. However, during the period of maximum LPS enhancement (8–11 days before irradiation), the RFC response for the LPS-treated irradiated mice reached a value of nearly 90% of the nonirradiated controls. Pretreatment of animals with LPS within this time period almost completely prevented the development of radiation-caused immunosuppression.

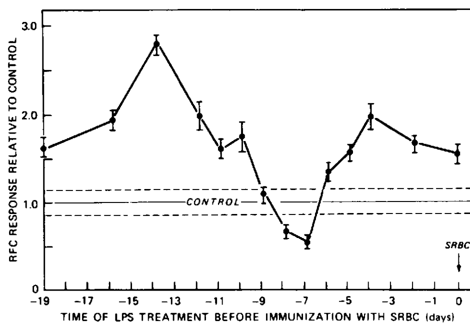

FIG. 1. The RFC response of ICR female mice injected with 10 μg of LPS (*S. marcescens*) at selected time intervals before immunization with 1×10^8 SRBC. Control value for RFC response to SRBC was $2.1 \pm .16 \times 10^4$ RFC per 10^6 spleen cells. Vertical bars represent ±1 SD.

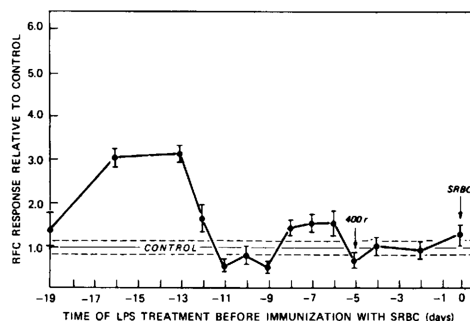

FIG. 2. The RFC response relative to control of ICR female mice injected with 10 μg LPS (*S. marcescens*) at selected time intervals with respect to irradiation (400 r) and immunization with 1×10^8 SRBC. The RFC response to SRBC in irradiated controls (without LPS) was $6.1 \pm .37 \times 10^3$ RFC per 10^6 spleen cells. Vertical bars represent ±1 SD.

Having observed the above described protective effect of LPS when such treatments preceded irradiation, our next aim was to determine if LPS treatment could induce the restoration of the RFC immune response in *previously* irradiated animals. Mice in groups of six were simultaneously immunized and treated with LPS 3 days after receiving 400 r whole-body irradiation (Table I). Comparison was made to one normal and one irradiated control group in order to assess the degree of enhancement of the RFC response in the LPS-treated irradiated group when compared to the irradiated control mice. This represents an immune response which is equivalent to 78% of the nonirradiated control group.

Effect of LPS on the PFC immune response to SRBC in irradiated mice. The results of the experiments in which the effect of radiation on the PFC response was prevented by application of LPS are summarized in Table II. Except for the animals in group 1, all mice showed significant enhancement of the PFC response as compared to animals irradiated and immunized but not LPS treated. The largest enhancement was observed for group 2, in which the LPS treatment preceded irradiation by 2 days and immunization by 7 days. If LPS treatment preceded irradiation by 2 days and immunization by 4 days (group 1 in Table II), the radiation-caused damage could not be prevented.

Discussion. Before beginning our discussion of experimental data it may be worthwhile to justify the use of pooled spleen cell-suspensions within our experimental design. The use of pooled spleen cells has the obvious disadvantage of obliterating the individual variations of animal responses within a given group. In preliminary studies we had determined that the standard deviation of the immune response within most groups was less than 10% of their means. In view of this high degree of consistency we felt justified in pool-

TABLE I. IMMUNE RESTORATION OF IRRADIATED (400 r) MICE BY LPS (*Serratia marcescens*) TREATMENT.

Treatment[a]	Radiation (400 r)	SRBC (1×10^7)	RFC's per 10^6 spleen cells ($\times 10^3$)	Relative to control No. 1	Relative to control No. 2
LPS (10 μg)	+	+	9.7 ± 1.4[b]	3.2 ± .88[c]	0.78 ± .20
Control No. 1 (no LPS)	+	+	3.0 ± 0.7	—	0.28 ± .05
Control No. 2	none	+	12.4 ± 1.2	4.13 ± 1.04	—

[a] Mice were simultaneously immunized and LPS-treated 3 days after irradiation.
[b] Significant at the 0.01 level.
[c] Represents ± 1 SD.

TABLE II. PROTECTIVE EFFECT OF LPS ON THE IMMUNE-RESPONSE OF X-IRRADIATED MICE IN THE PFC RESPONSE.

Group	LPS[a] (on day)	Radiation[b] (on day)	SRBC[c] (on day)	PFC's/10^6 spleen cells[d]	PFC Response Relative to Control[d]
1	0	2	4	2.56 ± 0.19	1.19 ± 0.11
	none	2	4	2.14 ± 0.11	
2	0	2	7	23.11 ± 0.62[e]	5.37 ± 1.11[e]
	none	2	7	4.1 ± 0.88	
3	0	2	14	15.2 ± 1.05[e]	2.38 ± 0.38[e]
	none	2	14	6.5 ± 0.95	
4	0	10	12	7.67 ± 0.57[e]	3.43 ± 0.83[e]
	none	10	12	2.17 ± 0.48	
5	0	10	15	8.65 ± 0.49[e]	4.32 ± 1.12[e]
	none	10	15	2.09 ± 0.53	
6	0	10	22	6.94 ± 0.78[e]	2.36 ± 0.33[e]
	none	10	22	2.93 ± 0.26	
Nonirradiated control				97.4 ± 7.81	

[a] 10 μg LPS ip per mouse.
[b] 400 r whole-body.
[c] 1×10^8 SRBC/mouse.
[d] ±1 SD.
[e] Significant at the 0.05 level.

ing the spleen cells of animals within a given group. The enormous time saving step of pooled cell suspensions provided us with the benefit of an experimental design in which all animals were irradiated on the same day and subsequently sacrificed and assayed on the same day.

Of the many mechanisms whereby endotoxin is supposed to affect the immune system, none offers a satisfactory explanation for the biphasic survival of lethally irradiated animals which we observed in our previous study (16), nor the biphasic RFC response (in normal and irradiated animals), reported here. In the latter assay the remarkable adjuvant effect of LPS given 8–11 days before the immunogen cannot be explained on the basis of any current theories on the mode of adjuvant action.

It is generally accepted that cell proliferation must be an essential feature of the early immune response (17), in which an early radiosensitive phase rapidly moves into a radioresistant phase (18). Endotoxin, by virtue of its antigenic and mitogenic property, may therefore stimulate the antibody-producing precursor cell (B-cell) to differentiate into the less radiosensitive plasma cell (19). It seems likely that LPS affects stem cells, and induces blastogenesis and differentiation, which establish a synchronous cell population undergoing a series of mitotic divisions characterized by cytological, functional and associated radio-resistant changes. That cell synchronization by endotoxin may play a key role in the biphasic protection pattern against lethal doses of radiation is substantiated by the observation that daily repeated LPS treat-

ments provide no radiation protection (20–22).

One cannot exclude the possibility that two different cell types are affected by LPS in both the radiation protection and in the adjuvant effects. One type responds to LPS-induced stimulation relatively quickly and reaches its optimal readiness for irradiation protection or immunization within a few days. The achievement of the same stage of activation takes 8–11 days for the second cell type.

At this time only the above and some additional theoretical possibilities can be offered to explain the multiphasic nature of the LPS-induced enhanced resistance to radiation and enhanced immune response. The great similarities in the two time curves indicate to us eventual interrelationships between the two phenomena, or at least similarities in the chain of events involved in both. Further experiments on the possible mode of action are in progress in our laboratories.

Another point for discussion is the difference in radiosensitivity as observed by the RFC and PFC assays. Although LPS pretreatment conveyed a several-fold increase in both the RFC and PFC response during optimal treatment times, a major difference concerning the radiosensitivities of these two immune responses exists. While a constant exposure dose of 400 r reduced the RFC response of irradiated animals to 30% of the nonirradiated controls, the same dose reduced the PFC response to only 3–7% of the PFC value of the normal, nonirradiated controls. Equally, a disparity exists for the optimally LPS-stimulated groups in their RFC and PFC response when compared to their nonirradiated controls. In contrast to the maximal LPS enhancement of RFC response which attained a value of nearly 90% of the nonirradiated controls, the highest LPS enhanced RFC response in irradiated animals was less than 24% of the nonirradiated controls. The assumption of two separate cell lines for rosetting and antibody production response would conveniently serve to explain the lack of parallelism of LPS radioprotection of the PFC and RFC responses as well as differences in radiosensitivity. It is known that the immunological role of T-cells in expressing cellular immunity is *not* markedly inhibited by irradiation in doses greatly affecting antibody formation (23, 24). That rosette-forming cells are sensitized immunocytes is well established by the simple demonstration that these cells drastically increase in numbers in the spleens of mice immunized with SRBC. However, their precise *in vivo* function as well as their relationship to antibody producing cells remains speculative and controversial. Neonie and Harris (25) postulate that the rosetting cell involves newly synthesized antibody molecules which are still attached to the cell surface and can act as specific receptors for antigen. It has also been shown that: (a) the *in vitro* development of rosette-forming cells requires an active synthetic process; (b) anti-IgG and IgM antibodies inhibit both RFC and PFC assays (26) and (c) RFC's are susceptable to the action of 2-mercaptoethanol to the same extent as IgM antibodies (27). The demonstration of these similarities between specific antibodies and the surface receptors of rosetting cells, however, is by no means conclusive proof that rosetting cells and antibody producing cells are one and the same. It is well known that T-cells also carry antigen-specific surface receptors (28) which are capable of antigen binding (29).

Convincing evidence that PFC's and RFC's are largely independent populations comes from the work of Wilson (30) who was able to separate RFC's from PFC's by means of velocity sedimentation. More convincing were his results obtained by using isolated individual rosette forming and plaque forming cells. As he found, very few RFC's were able to produce plaques. Equally, few PFC's were able to form rosettes. It must also be pointed out that the kinetic profile of the RFC response is entirely different from the PFC response suggesting that RFC's are long-lived immunocytes or memory cells (26).

The final point we wish to emphasize is that it is possible to compensate for radiation-caused immune damage by LPS, but only if LPS is applied at the proper time relative to irradiation. The practical use of such pretreatment in patients undergoing radiotherapy is an obvious aim. How these observations will apply to human cases remains to be explored.

Summary. It has been established that endotoxic lipopolysaccharide (LPS) exerts a

long-term effect on the immune system. This effect is multi-phasic. Contingent upon the time interval between adjuvant application and immunogen injection, either enhancement or immunosuppressive effect could be observed. LPS given 2–4 or 10–16 days before sheep red blood cell (SRBC) immunogen significantly enhanced the immune response as measured by rosette formation (RFC). LPS given 7 or 8 days before SRBC was immunosuppressive.

Based on this information, attempts were made to prevent or repair radiation-caused immunosuppression by using LPS at optimal time. It was found that under such conditions LPS can minimize such damage. The application of LPS 3 days after irradiation was similarly effective in the repair of immunosuppression as measured by the RFC assay. LPS was less efficient but still useful in reducing the radiation-caused suppression of the number of plaque forming cells (PFC).

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