

Amplification of Migratory Inhibition Factor Production During the First 48 Hours of Exposure to Antigen

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When lymphocyte-macrophage suspensions from sensitized animals are preincubated with specific antigen for 24 or more h, the following results are observed. (i) In a standard capillary macrophage migration test, there is complete inhibition of migration. (ii) When the preincubated cell suspension is mixed in varying proportions with a similar suspension from nonsensitized animals and a macrophage migration test is performed, there is no linear relationship between the degree of inhibition of migration and the proportion of sensitized lymphocytes initially present. Inhibition thus appears to be an "all-or-none" effect. (iii) In spite of the second observation, increasing periods of preincubation with antigen result in increasing inhibition. (iv) These results suggest the existence of a complex amplifying mechanism operating within the early period of exposure to antigen. (v) To test the possibility that cell proliferation contributes to this amplification, cells from sensitized guinea pigs were irradiated with a dose of 1,000 rads prior to preincubation with antigen. Despite this dose, which virtually abolishes cell division in other systems, no diminution whatever in the amplification of inhibition was observed. These results suggest the existence of an early phase of increased production of migratory inhibition factor that is not dependent on cell division but that may be related to "recruitment" of nonsensitized lymphocytes.

Elucidation of cell-mediated immunity has recently been facilitated by the development of several in vitro techniques which allow delineation of the important role of lymphocyte-macrophage interactions (3, 5). When lymphocytes from sensitized animals are cultured with the appropriate antigen, lymphoblasts begin to appear after 24 h of incubation, but lymphocyte proliferation has been demonstrated only after the first 72 h (8). However, early proliferation has not been excluded, and other mechanisms such as "recruitment" of nonsensitized lymphocytes may also be operating within the first 3 days of exposure to antigen (8).

These reported studies seemed relevant to our own observations on inhibition of macrophage migration, which consistently showed better delineation between positive and negative results at 48 and 72 h compared with that at 24 h. It is likely that the net area of macrophage

migration is determined by the resultant of two opposing forces, namely, the initial rate of macrophage migration (i.e., "macrophage bolting") and the rapidity with which the sensitive lymphocyte population can achieve inhibitory concentrations of migration inhibitory factor (MIF) in the culture medium. The fact that the difference between positive and negative inhibition tests is more marked at 48 to 72 h than at 24 h, despite "macrophage bolting," suggested increased production of MIF after the first 24 h. Consequently, it was decided to observe the kinetics of inhibition of macrophage migration by lymphocytes previously exposed to specific antigen for intervals up to 2 days.

One possible mechanism for increased MIF production is cell proliferation that results in an increased number of lymphocytes producing MIF. To test this possibility, it was decided to irradiate the sensitive lymphocyte population prior to preincubation with antigen. In other oxygenated, mammalian cell populations, a dose of 1,000 rads has been shown to have the

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advantage of affecting a 99% reduction in cell division, while having an insignificant effect on cell function (1, 4, 9, 10). It is likely, therefore, that this dose of radiation will at least diminish (or perhaps even abolish) any amplification of MIF production that is dependent on cell division. At the same time, this dose should have little (or no) effect on "recruitment" or on any enzyme-inductive mechanisms, both of which might also be necessary for MIF production (1, 9, 10).

MATERIALS AND METHODS

The general design of the experiments was as follows. To provide sufficient cells for manipulation and to reduce differences between individual animals, mineral oil-induced peritoneal lymphocyte-macrophage suspensions were obtained from two or three tuberculin-sensitive guinea pigs. Preservative-free tuberculin purified protein derivative (PPD), at a concentration of 32 $\mu\text{g}/\text{ml}$, was added to a pooled cell suspension. After intervals of 0, 1, and 2 days of preincubation with antigen, samples of this suspension were mixed with similar pooled suspensions of lymphocytes and macrophages obtained from nonsensitized guinea pigs. In each case, the ratio of sensitized to nonsensitized cells varied between 1:0 and 1:180. The resultant mixed cell suspensions were then placed in capillary tubes within Mackness chambers, and the areas of macrophage migration were measured 48 h thereafter. By this means, it was possible to compare the capacity of the lymphocyte population(s) to inhibit macrophage migration after the various intervals of preexposure to antigen. To exclude the possibility of inhibition of migration as a result of any other factors, antigen-free controls were also set up in each experiment.

For the radiation studies, one-half of the pooled cell suspension was immediately subjected to 1,000 rads of radiation before the addition of PPD. The remainder of the cell suspension was not irradiated. At intervals of 0, 1, and 2 days, the irradiated cell suspension was mixed with pooled peritoneal exudate cells from nonsensitive guinea pigs in ratios varying between 1:0 and 1:180. The same procedure was followed for the nonirradiated cell population. The areas of macrophage migration were measured 48 h thereafter and compared with areas of migration achieved by pooled nonsensitive cell suspension.

Guinea pigs. Female Hartley guinea pigs (approximately 400 g in weight) were used as the source of peritoneal exudate cells.

Immunization. Complete Freund adjuvant (0.1 mg; Difco H37 Ra) was injected into each hind footpad approximately 2 to 3 weeks before the experiment. Skin testing with tuberculin PPD (Parke, Davis & Co) was performed to insure adequate sensitivity.

Collection and treatment of cells. For each experiment, two or three control and sensitized animals were used to guarantee an adequate yield of cells for manipulation. Sterile, light mineral oil (20 ml) was injected intraperitoneally 72 h before the experiment to evoke a cellular exudate. After sacrifice, the perito-

neal cavity was exposed by a midline incision and was washed out with Hanks balanced salt solution. The resulting cell suspension was collected into 50-ml polypropylene tubes which were centrifuged for 10 min at 1,200 rpm, and the supernatant fluids were discarded. The cells from the two or three animals were then pooled and washed twice with 50 ml of cold Hanks solution. After the final wash, the cells were suspended in 10 ml of Eagle medium containing 15% heat-inactivated guinea pig serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cell and viability counts were performed by using the Trypan blue exclusion technique, and the suspensions were adjusted to 20,000 viable cells/ mm^3 .

Preincubation of cells with antigen. The cell populations from the tuberculin-sensitive animals were preincubated for 0, 24, and 48 h at 37 C in the presence of 32 μg of preservative-free, tuberculin PPD (Parke, Davis, & Co., lot 974776F) per ml. After this, the cell suspensions were centrifuged and the supernatant fluids were removed. On the basis of viability counts, the cells were then resuspended to a concentration of 20,000/ mm^3 . From these suspensions, the various ratios of sensitive to nonsensitive cells were made in the range of 1:0 to 1:180, respectively.

Radiation. A model M Gammator (Radiation Machinery Corp.) was used with a source material of cesium-137 chloride, a source strength of 800 Ci $\pm 5\%$, and a gamma energy of 0.662 meV. The dose rate was equal to 66.2 Krads/h and, 1,000 rads were given to the cells over 0.91 min.

Macrophage migration inhibition technique. The basic method of David (2) was used. Briefly, 50- μl iter capillary tubes (internal diameter 1.1 mm) were filled with the various cell suspensions, sealed at one end with paraffin, centrifuged for 5 min at 800 rpm, cut at the cell-fluid interface, and placed in Mackness chambers. Two chambers (each containing three capillaries) were made for each cell "dilution," and Eagle medium containing serum, antibiotics, and 32 μg of PPD per ml was introduced. Antigen-free control chambers were also set up. The cells were then allowed to migrate for 48 h at 37 C before their areas of migration were projected, traced, and quantified by planimetry. Analysis of variance and regression analysis were used for statistical examination of the results.

RESULTS

The areas of macrophage migration for the different cell dilutions after 0, 24, and 48 h of preincubation with antigen are contained in Table 1 and are shown diagrammatically in Fig. 1 to 3. In Fig. 4, for purposes of comparison, the results are expressed as percentage inhibition of the controls for each period of preincubation with antigen, i.e., 0, 24, and 48 h.

Examination of Fig. 1 to 3 suggests, first, that increasing the period of preincubation with antigen increases the ability of the lymphocyte population(s) to inhibit macrophage migration. Visual inspection suggests that significant inhi-

TABLE 1. Areas (cm²) of macrophage migration (mean ± 2 SE) after 0, 1, and 2 days of preincubation with antigen

Cell dilutions	Days		
	0	1	2
Control (C) cells	9.2 ± 0.7	6.4 ± 1.1	6.3 ± 0.6
Sensitized (S) cells	1.8 ± 0.1	0	0
S/20	2.9 ± 0.2	3.0 ± 0.2	5.0 ± 0.1
S/40	5.8 ± 0.8	4.9 ± 0.2	5.9 ± 0.2
S/60	4.9 ± 0.7	3.9 ± 0.1	5.1 ± 0.1
S/80	4.0 ± 0.7	2.7 ± 0.3	5.3 ± 0.1
S/100	5.0 ± 1.2	5.3 ± 2.8	4.7 ± 0.1
S/120	4.3 ± 0.4	7.3 ± 0.5	6.0 ± 0.2
S/140	5.3 ± 0.5	6.6 ± 2.1	7.8 ± 1.5
S/160	5.9 ± 1.2	6.3 ± 0.4	7.2 ± 0.2
S/180	5.7 ± 1.0	6.8 ± 0.9	8.7 ± 0.8

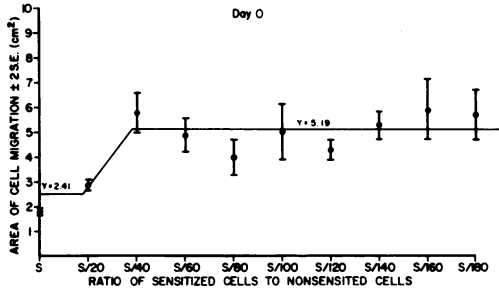


FIG. 1. Inhibition of macrophage migration. Day 0 represents no preincubation of antigen with "sensitive" cells (peritoneal exudate cells of sensitized animals) prior to dilution with "nonsensitive" cells. Areas of cell migration were measured at 48 h after being placed in Mackness chambers.

bition occurred up to a dilution of 1:20 of the sensitized cells with no preincubation with antigen, up to 1:80 after 24 h preincubation, and 1:120 with 48 h preincubation. Second, the patterns of migration after each period of preincubation are suggestive of an "all-or-none" effect, with a sharp transition between inhibition and noninhibition and with no linear regression (beta = 0) within these two modalities (i.e., "all" and "none"). These impressions are consistent with the results of anova regression analysis of the data. This shows that, after zero preincubation time, there is no linear regression of the area of macrophage migration with the various cell dilutions ($P > 0.5$). However, there is a straight line of zero slope (beta = 0; $Y = 2.41$) joining points S/0 and S/20 and a significantly different ($P < 0.5$) straight line ($Y = 5.19$), also of zero slope, between S/40 and S/180. A similar picture holds after 24 h of

preincubation (Fig. 2) with no straight line through all the points ($P > 0.5$). However, in this case, there is a straight line ($P < 0.5$) between S/20 and S/80 ($Y = 3.19$) and a different ($P < 0.5$) straight line between S/100 and S/180 ($Y = 6.50$). After 48 h of preincubation (Fig. 3), there is, again, no overall straight line through all the points ($P < 0.5$). On this

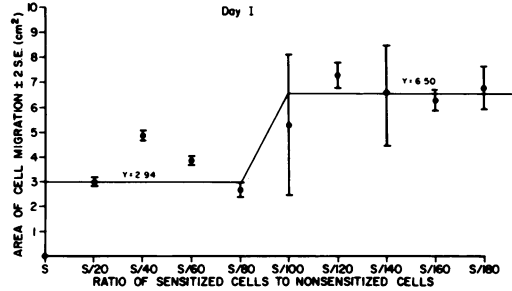


FIG. 2. Inhibition of macrophage migration. Day I represents 24 h of preincubation of antigen with "sensitive" cells prior to dilution with "nonsensitive" cells. Areas of cell migration were measured at 48 h after being placed in Mackness chambers.

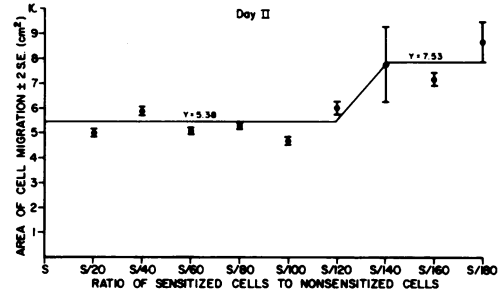


FIG. 3. Inhibition of macrophage migration. Day II represents 48 h of preincubation of antigen with "sensitive" cells prior to dilution with "nonsensitive" cells. Areas of cell migration were measured at 48 h after being placed in Mackness chambers.

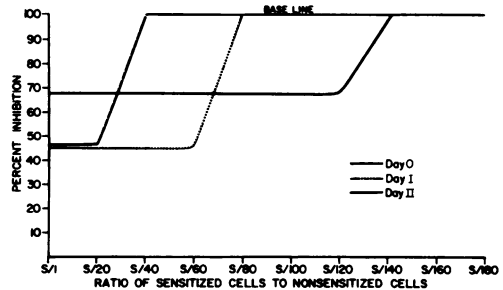


FIG. 4. Inhibition of macrophage migration. Results are expressed as percentage inhibition of controls for each period of preincubation with antigen.

occasion, there is a straight line between S/20 and S/120 ($Y = 5.38$) and another distinct line between S/140 and S/180 ($Y = 7.53$).

It should be noted that, after 24 and 48 h of preincubation with antigen, the undiluted sensitized cells (S/0) showed no migration whatever. This is probably due to prior immobilization of the macrophages by MIF elaborated during the preincubation period. For this reason, these two results are excluded from the analyses. This also means that the macrophages from the nonsensitive animals are probably responsible for all the observed migration in the mixed cell suspensions.

In the antigen-free control chambers (i.e., where the sensitive lymphocytes were preincubated without PPD prior to being mixed with nonsensitive cells), inhibition of macrophage migration was never observed. This, along with the fact that pooled suspensions from several animals were employed for all the manipulations, suggests that a mixed leukocyte reaction was not responsible for the observed patterns of inhibition of migration (Table 2).

The mean areas of macrophage migration for irradiated and nonirradiated cells are shown in Table 3. After zero preincubation time, there was significant ($P < 0.5$) inhibition of macrophage migration in the undiluted sensitized cell suspensions (S/0) only. Prior irradiation (1,000 rads) had no effect on this pattern ($P > 0.5$). There was no inhibition with cell "dilutions" between S/20 and S/180 in either the irradiated or nonirradiated groups ($P > 0.5$). Therefore, after zero preincubation with antigen, 1,000 rads of radiation made no difference to the pattern or areas of migration.

After 24 h of preincubation with antigen, it can be seen from Table 3 that, as in the first experiment (Table 1), the undiluted sensitive cell suspensions, whether irradiated or not,

undergo no migration whatever. Also, in contrast with the pattern observed in the zero preincubation experiment, there is significant ($P < 0.5$) inhibition of macrophage migration in all cell dilutions up to S/180 in both the irradiated and nonirradiated groups. Furthermore, statistical analysis shows that there is no overall difference ($P > 0.5$) between the mean area of migration of the irradiated and nonirradiated groups. An identical picture holds after 48 h of preincubation of the sensitive cells with antigen, i.e., no migration whatever with the undiluted sensitive cell suspensions, significant ($P < 0.5$) inhibition of macrophage migration in all "dilutions" up to S/180, and no statistically significant difference ($P > 0.5$) between the overall mean areas of migration of the irradiated and the nonirradiated cell groups.

Thus, as in the previous cell "dilution" experiment (Table 1), there was greater inhibition of macrophage migration after 24 h of preincubation with antigen compared with that observed after no preincubation. In this particular experi-

TABLE 2. Areas (cm^2) of macrophage migration in chambers with and without antigen

Cell dilutions	Area of macrophage migration (cm^2)	
	Without PPD ^a	With PPD
Control (C) cells ^b	7.2 ± 1.1	7.7 ± 1.7
Sensitive (S) cells ^b		
S/5	7.0 ± 2.5	2.8 ± 1.0
S/40	6.8 ± 2.1	2.6 ± 1.0
S/80	7.2 ± 1.4	3.4 ± 1.2
S/100	7.0 ± 3.0	7.0 ± 2.1
S/160	7.9 ± 1.4	6.2 ± 3.2
S/320	6.9 ± 2.8	7.4 ± 1.5

^a PPD, Purified protein derivative.

^b Control and sensitized cells represent pooled suspension from several (2 to 3) animals.

TABLE 3. Areas (cm^2) of macrophage migration (mean ± 2 SE) after 0, 1, and 2 days of preincubation with antigen with and without prior irradiation (1,000 rads)

Cell dilutions	0 Days		1 Day		2 Days	
	Irradiated	Nonirradiated	Irradiated	Nonirradiated	Irradiated	Nonirradiated
Control cells	10.9 ± 0.64	7.8 ± 0.18	7.8 ± 0.34	0.3 ± 0.74	4.5 ± 0.32	5.3 ± 0.92
Sensitive (S) cells	1.3 ± 0.02	1.6 ± 0.04	0	0	0	0
S/20	6.1 ± 0.34	6.4 ± 0.18	1.6 ± 0.34	1.4 ± 0.04	2.8 ± 0.08	1.1 ± 0.02
S/40	8.3 ± 0.20	6.5 ± 0.28	1.9 ± 0.06	2.3 ± 0.61	3.8 ± 0.24	1.6 ± 0.14
S/60	8.0 ± 0.32	7.4 ± 0.28	1.1 ± 0.06	2.1 ± 0.06	2.9 ± 0.18	2.2 ± 0.06
S/80	7.7 ± 0.48	7.2 ± 0.46	0.9 ± 0.06	1.8 ± 0.18	3.5 ± 0.12	2.2 ± 0.18
S/100	8.1 ± 0.16	9.2 ± 0.60	0.8 ± 0.08	1.8 ± 0.42	2.6 ± 0.02	4.1 ± 0.14
S/120	7.0 ± 0.74	8.3 ± 0.24	2.5 ± 0.22	1.9 ± 0.10	2.0 ± 0.02	2.7 ± 0.12
S/140	6.4 ± 0.36	7.2 ± 0.06	2.4 ± 0.20	3.2 ± 0.06	3.8 ± 0.18	3.6 ± 0.14
S/160	8.0 ± 0.18	7.3 ± 0.34	2.2 ± 0.02	1.8 ± 0.22	2.8 ± 0.08	3.4 ± 0.32
S/180	8.0 ± 0.20	8.1 ± 0.22	3.1 ± 0.10	4.0 ± 0.82	3.4 ± 0.54	3.9 ± 0.28

ment (Table 3), there was no further increase in inhibition after 48 h of preincubation compared with that seen after 24 h of preincubation. These results are consistent with the first observation (Table 1) that preincubation with antigen amplifies the ability of a sensitive lymphocyte population to inhibit macrophage migration. It has also been shown that this amplification is not inhibited by 1,000 rads of radiation (Table 3).

DISCUSSION

The above results are consistent with the following conclusions. First, preincubation with specific antigen of a lymphocyte-macrophage suspension from sensitive animals for a period of 24 h or more results in complete inhibition of macrophage migration when this suspension is then placed in capillary tubes. Presumably, this is due to immobilization of the macrophages during the preincubation period so that they are thereafter totally unable to migrate. In this case, the element of "macrophage bolting" defined in the introduction has been eliminated, resulting in a discrete test yielding only positive or negative results. This effect may be related to the macrophage aggregation phenomenon described by Lolekha, Dray, and Gotoff (7).

Second, statistical analysis of the results fails to show a dose-response relationship between inhibition of macrophage migration and the number of sensitized lymphocytes initially present. Indeed, inhibition appeared to be an "all-or-none" effect. This is surprising in view of the third observation that increasing the time of preincubation with PPD resulted in an increase in the ability of the total lymphocyte populations to inhibit macrophage migration as measured by the "cell dilution" technique. This increase in inhibition along with the "all-or-none" effect suggests the existence of a rather complex amplifying mechanism operating during the first 24 to 48 h of exposure to specific antigen. Marshall, Valentine, and Lawrence suggested that cell proliferation, which might explain amplification, is probably absent during this early period, although their experiments did not specifically exclude this (8).

Consequently, the second experiment (Table 3) was designed to test the hypothesis that cellular proliferation is not responsible for the amplification of the ability of lymphocytes to inhibit macrophage migration during this first 24 to 48 h of incubation with specific antigen. In other oxygenated mammalian cell systems studied *in vivo* or *in vitro*, 1,000 rads of radiation reduces cell division by over 99% and has a

minimal effect on cell function (1, 4, 9, 10). The fact that in this experiment 1,000 rads of irradiation had no effect on the observed amplification of inhibition is consistent not only with these radiobiological reports but also with the more direct observations of Marshall, Valentine, and Lawrence (8). It is therefore reasonable to assume that cellular proliferation is not responsible for the amplification of MIF production observed during the first 24 to 48 h of preincubation with antigen.

These data together suggest the possible existence of two phases of amplification when sensitive lymphocytes are exposed to specific antigen. The "early phase" (0 to 3 days) is associated with increased production of MIF and is not dependent on cell division. The "late phase" (4 to 7 days), in which lymphocyte proliferation does occur, has been shown to be associated with target cell destruction (8). This picture is also consistent with the observations of Brent and Medawar (1) on both the "immune lymphocyte transfer reaction" (ILT reaction) and the "normal lymphocyte transfer" (NLT reaction). In both the ILT and NLT reactions, where the homologous lymphocytes are injected into the skin of a recipient, there is an early inflammatory reaction during the first three days and a later flare-up during the 5th to 7th day. The "early phase" is radioresistant (to 1,000 rads), and the "late phase" is radiosensitive. These findings were taken by Brent and Medawar (1) to imply that cell division had a role in the "late phase" reaction only. The parallel between these and our own observations is clear.

Two other recognized mechanisms which may underlie the phenomenon of "early phase amplification" and which might be expected to be radioresistant (1, 9, 10) are "recruitment" (6) or the time necessary for enzyme induction related to the production of MIF or both. Further kinetic studies will be necessary to define these possibilities.

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