THE MEDIATOR OF CELLULAR IMMUNITY

IX. The Relationship between Cellular Hypersensitivity and Acquired Cellular Resistance in Rats Infected with *Listeria monocytogenes**

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A parallel in the development of cellular hypersensitivity and acquired cellular resistance has been observed in infections caused by intracellular bacterial parasites, protozoa, and certain viruses (1), and provides one of several arguments that delayed-type hypersensitivity (DTH)[1] and resistance are but different expressions of the same cell-mediated response to microbial antigens (2). At the cellular level, it has been demonstrated that DTH and cellular resistance are mediated by thymus-dependent (T) cells (3-7) and that both depend for their full expression upon the activity of macrophages (8-10).

The mechanism by which macrophages and sensitized lymphocytes collaborate in the host's defense has not been fully elucidated; however, there are reasons for thinking that meaningful interactions occur locally in foci of infection. It may be significant therefore that lymphocytes obtained from inflammatory exudates can be stimulated by antigens to release soluble products that influence macrophages in various ways (11). The results of the current investigation encourage the belief that at least one of these products, macrophage migration inhibitory factor (MIF) is formed by lymphocytes which are recruited from the blood in response to inflammation. It will be shown that the localization of MIF-producing cells in the inflamed peritoneal cavity of rats infected with *Listeria monocytogenes* coincides or closely overlaps the arrival from the circulation of lymphocytes which can protect normal recipients against a *Listeria* challenge. These, and other findings, point to a functional link between cellular hypersensitivity and cellular resistance to infection, and give credence to the view that MIF has a purposeful role in the expression of cellular immunity.

Materials and Methods

Animals. Male and female (Lewis × DA)F_1 hybrid rats were used. Most were 8- to 12-wk old and weighed 160–300 g; however, antimicrobial resistance was measured in animals that were 5-wk old and weighed 70–90 g.

Microorganisms. *L. monocytogenes*, strain EGD, was used for immunization and challenge. A suspension of the organisms, prepared from a 16 h trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md.) culture of infected spleen, was stored in liquid nitrogen and recovered as needed. Homogenates of infected liver and spleen were plated on tryptic soy agar

* Supported by grant IM-6D from the American Cancer Society and grant CA 15420 from the National Cancer Institute.

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[1] *Abbreviations used in this paper:* DTH, delayed-type hypersensitivity; LM, *Listeria monocytogenes;* MIF, migration inhibitory factor; PEC, peritoneal exudate cells; TDL, thoracic duct lymphocytes.

(Baltimore Biological Laboratories). *Francisella tularensis*, strain LVS, was passaged in rats, recovered from infected spleen, and grown in liquid medium (12). The organisms were killed by heating a saline suspension (10^9/ml) for 1 h ar 60°C.

Antigen. *L. monocytogenes* (LM) antigen was prepared by a modification of the method described by Hinsdill and Berman (13). An 18 h culture of the organisms was killed by phenol. The dead bacteria were washed once with 0.85% saline and thrice with distilled water. They were then suspended in distilled water and sonicated for 20 min in an ice bath using a Biosonik probe (20 kcycles, 300 W, Bronwill Scientific, Rochester, N. Y.) operating at 10% output. The suspension of ruptured cells was centrifuged for 30 min at 18,000 *g*. The supernate, containing soluble antigens of the parasite, was then sterilized by Millipore filtration and lyophilized.

Immunization of Rats and Measurement of Protective Immunity. Rats were immunized with approximately 5×10^6 living LM. The organisms were suspended in 0.6 ml of saline and injected subcutaneously at multiple sites in the hind quarters: both hind foot pads, the base of the tail, and the lower abdomen. The immunity engendered by this procedure was measured indirectly in terms of the resistance conferred on normal recipients by an injection of thoracic duct lymphocytes (TDL) or peritoneal exudate cells (PEC) obtained from infected donors (14).

Cells. In most experiments, TDL were collected for 16–20 h into heparinized Ringer's solution without added antibiotics. In some experiments, however, the cells were collected in Ringer's solution containing 100 IU/ml of penicillin. PEC were obtained from rats which had been stimulated intraperitoneally with 1 ml of saline containing 10^7 killed *F. tularensis*. A population of cells rich in macrophages was obtained from the peritoneal cavities of normal rats 72 h after the animals had been stimulated intraperitoneally with 3 ml/100 g body weight of 12% sodium caseinate. The techniques for inducing exudates and harvesting the cells have been described elsewhere (15).

MIF Assay. The MIF assay described in an earlier report (16) was modified with the express purpose of comparing the activity of TDL and PEC obtained from individual donors. To this end, lymphocytes issuing from the thoracic duct 20–24 h after incannulation, or PEC harvested at different intervals after stimulation with killed *F. tularensis*, were combined in different proportions with casein-induced "indicator" cells. These were obtained from several normal rats. The cell mixtures were packed into capillary tubes that were then fixed to the floor of disposable culture chambers (Sterilin Ltd., Richmond, Surrey, England). Medium 199 containing 15% heat-inactivated rat serum and different amounts of LM antigen were added to the chambers before the latter were sealed. After incubation for 24 h at 37°C, the area of cell migration from the individual capillaries was measured by projection microscopy and planimetry. Cell migration in the presence of antigen was expressed as percent migration of a sample of the same cell mixture cultivated in antigen-free medium.

DTH. The radiometric method described by Lefford (17) was used to measure DTH to LM antigen. Test subjects were given a single pulse of 0.5 μCi/g of [^{3}H]thymidine (3 Ci/mmol, New England Nuclear, Boston, Mass.). 24 h later, 1 μg of LM antigen in 0.02 ml of saline and 0.02 ml of saline diluent were injected into the pinna of the right and left ear, respectively. 24 h after the injection, a uniform tissue sample was removed from each injection site with a 6 mm skin biopsy punch. The individual samples were digested for 18 h at 50°C in 0.5 ml of NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill). and an equal volume of toluene. 20 μl of glacial acetic acid and 10 ml of PCS scintillant (Amersham/Searle Corp.) were then added and the samples stored at 4°C for 48 h. Radioactivity was measured in a scintillation spectrometer. The ratio of radioactivity in the right ear sample and left ear sample from individual rats was expressed as a "DTH index".

Statistical analysis. Differences between means of paired groups were evaluated by the *t* test. When more than two groups were compared, analysis of variance was applied and differences between means evaluated by the least significant difference method (18). The relative protection conferred on normal recipients by TDL and PEC from infected donors was determined by parallel line analysis (19).

Results

Kinetics of the Cellular Response to LM. Rats infected with LM develop cellular hypersensitivity to *Listeria* antigens and an accompanying state of cellular resistance to infection.

DTH. The development of DTH was measured quantitatively and kinetically in rats infected with 4.48×10^6 living *Listeria*. At predetermined intervals after infection, ear tests with 1 μg of LM antigen, were performed on groups of five animals. The results in Fig. 1 indicate that DTH was first detected 5 days after infection. Sensitivity increased rapidly to a maximum on day 6 then declined to a low but still significant level that persisted for at least 20 days.

IN VITRO EVIDENCE OF CELLULAR HYPERSENSITIVITY. The MIF assay was used to detect specifically sensitized lymphocytes in the inflamed peritoneal cavity of another group of similarly infected subjects. At predetermined intervals after infection, five to six rats were stimulated intraperitoneally with a saline suspension of killed *F. tularensis*. PEC (approximately 3×10^7) obtained from individual donors 24 h after stimulation were combined in vitro with 3×10^7 casein-induced indicator cells. The cell mixtures were packed into capillary tubes and cultivated in vitro in either antigen-free medium or medium containing 1 μg/ml of LM antigen.

Fig. 1 indicates that MIF-producing cells were first detected in exudates on the 5th day of the infection. Thereafter, their activity increased and later declined in concert with DTH as measured by the ear test. It is to be noted, however, that MIF-producing cells could not be detected in exudates induced 10 days or longer after infection, at a time when a DTH reaction could still be elicited in the intact animal.

GENERATION OF PROTECTIVE LYMPHOCYTES. TDL from *Listeria*-infected rats can protect normal recipients against a challenge infection with this organism (14). It was of interest therefore to determine the relationship between delivery of protective cells to the thoracic duct and the development of cellular hypersensitivity to LM antigen. The problem was studied by incannulating donor rats at

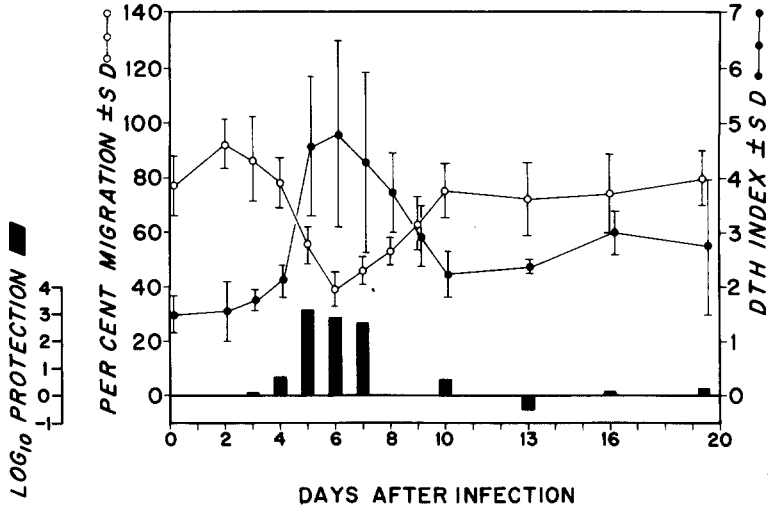

FIG. 1. Composite graph illustrating the relationship in *Listeria*-infected rats between the development of DTH (●—●), PEC migration inhibition (O—O), and the delivery to thoracic duct lymph of cells which can confer specific antimicrobial resistance on normal recipients (bar graph). Means of 5–6 per point ± SD.

different intervals after an immunizing *Listeria* infection. Cells issuing from the thoracic duct during the first 24 h of lymph drainage were transferred intravenously into syngeneic recipients. The cells, in a dose equal to 2×10^6/g of body weight, were transfused 1 h after the recipients had been challenged intravenously with approximately 3×10^6 LM.

As there was an unavoidable difference in the number of organisms in the challenge inocula, the results have been expressed in Fig. 1 as the mean difference 48 h after challenge in the number of viable units in the spleens of five adoptively immunized subjects and groups of five nonimmunized controls. The results indicate that protective lymphocytes were first demonstrated in the lymph 4 days after infection—i.e., immediately before cellular hypersensitivity could be detected. After day 5, the activity of the protective cells paralleled the level of cellular hypersensitivity as measured in the MIF assay.

Peritoneal exudates induced in *Listeria*-infected rats also contain cells which can immunize adoptively. Since residential cells in the peritoneal cavity of infected but otherwise unstimulated donors afford little if any protection (15), it follows that the protective cells originate elsewhere and localize in the peritoneal cavity in response to inflammation. This raises the question regarding the origin of the MIF-producing cells in exudates. Are they too members of an immigrant cell population and, if so, are they lineally related to the protective cells in central lymph? Evidence to support this proposition was obtained in the following experiment in which cells obtained from thoracic duct lymph and induced peritoneal exudates were compared with respect to their protective power and capacity to release MIF.

Protective Activity and MIF-Producing Capacity of TDL and PEC. Cytokinetic studies have shown that maximum resistance to LM is transferred by TDL and PEC obtained from donor rats on the 6th and 7th days of an immunizing infection, respectively (20). The protective capacity of the cells, and their response to LM antigen in vitro, were compared in an experiment that could be conveniently performed only by dividing it into two parts. In the first part, TDL obtained from 6-day *Listeria*-immune donors were transferred into normal recipients 1 h after the latter had been challenged intravenously with 1.70×10^6 LM. In the second part, PEC induced on day 6 and harvested on day 7 were given to animals that were challenged with 3.12×10^6 organisms.

The results in Fig. 2 indicate that "immune" TDL and "immune" PEC afforded protection in proportion to the number of cells transferred. However, PEC were more potent. Since the lineal regression of immunity on cell dose was not significantly different for the two donor cell populations, their protective capacities were compared by parallel line analysis. It was calculated that the protective power of PEC exceeded that of TDL by a factor of 7.7 and 3.8 when assayed in the liver and spleen, respectively.

TDL and PEC from the same groups of infected donors were tested in the MIF assay. To this end, mixtures containing different proportions of test cells and indicator cells were cultivated in the presence or absence of LM antigen. Table I shows that the concentration of antigen (1 μg/ml) used in this experiment had a modest effect on cell migration regardless of whether the cultures contained normal or immune cells. An effect ascribed to MIF was revealed in the

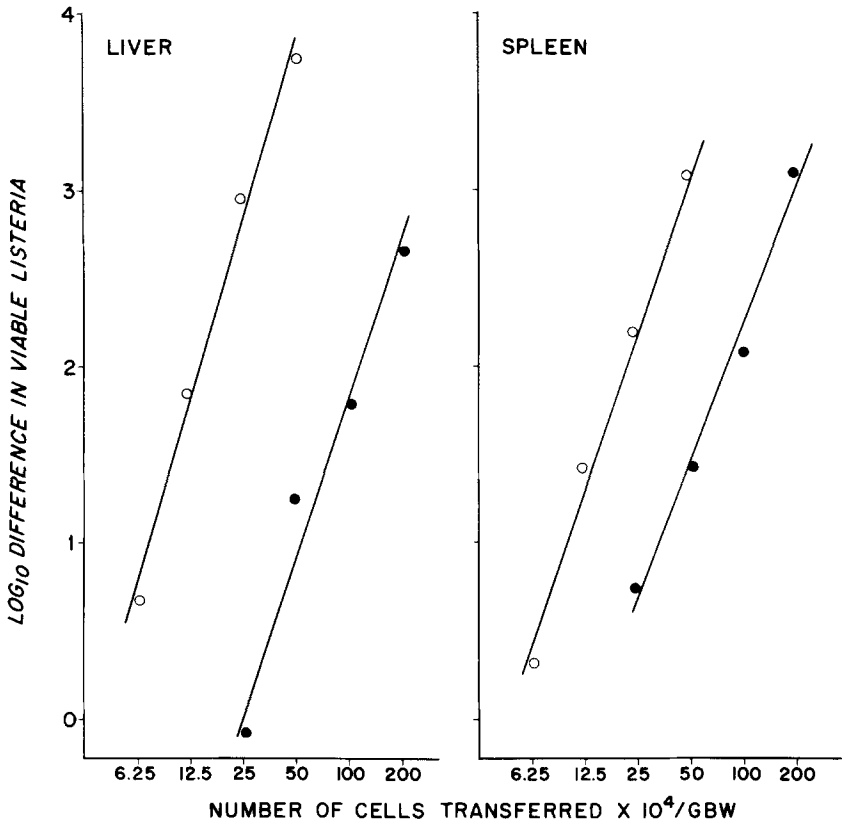

FIG. 2. Regression lines describing the immunity transferred adoptively by TDL (●—●) and PEC (O—O) obtained from donor rats 6–7 days after an immunizing *Listeria* infection. The TDL were collected 20–24 h after incannulation, while the exudate cells were harvested 24 h after the donors had been stimulated intraperitoneally with 10^7 killed *F. tularensis*. The cells were transferred intravenously 1 h after the recipients had been challenged intravenously with LM. Each point represents the mean difference 48 h after challenge in viable organisms in the tissues of five recipients of immune cells and five recipients of cells from normal, nonimmunized donors. The immunity transferred by PEC exceeded that transferred by TDL by a factor of 7.7 and 3.8 in the liver and spleen, respectively. GBW, gram body weight.

substantially greater inhibition observed in cultures containing 10% or more of immune PEC. The migration of cell mixtures containing 1% immune PEC and 99% indicator cells was not significantly different from their respective controls, a finding that accords with similar studies in the guinea pig (21). Cultures containing PEC were more responsive than those containing TDL. In the presence of LM antigen, the latter were inhibited, but only when the cultures contained the highest proportion (50%) of immune lymphocytes.

These findings indicate that PEC outperform TDL in both their protective capacity and ability to release MIF. If a single population of sensitized lymphocytes is responsible for these activities, one might expect that similar conditions would govern their tissue disposition in the body. That this in fact is the case, at least with respect to the exudate-seeking capacity of the cells concerned, was demonstrated in *Listeria*-infected rats by measuring the arrival

TABLE I
Response of PEC and TDL to LM Antigen in the MIF Assay

Capillary tube cultures*		Cell migration‡			
		Immune donors§		Normal donors	
Donor cells	Indicator cells	PEC	TDL	PEC	TDL
%	%				
50	50	54 ± 8‖	67 ± 7¶	88 ± 6	81 ± 10
25	75	55 ± 6‖	69 ± 8	86 ± 16	75 ± 6
10	90	55 ± 11‖	78 ± 10	85 ± 8	80 ± 8
1	99	71 ± 15	75 ± 9	87 ± 11	81 ± 11

* Cells obtained from thoracic duct lymph or peritoneal exudates harvested 24 h after stimulation were mixed in different proportions with casein-induced indicator cells.

‡ Migration of cells in the presence of 1 μg/ml LM antigen expressed as percent migration of the same cells in antigen-free medium. Means of 4–6 ± SD.

§ Cells obtained 6–7 days after the donors were infected with approximately 5×10^6 LM.

‖ Significantly different ($P < 0.01$) when compared with cells from normal donors.

¶ Significantly different ($P < 0.05$) when compared with cells from normal donors.

in the inflamed peritoneal cavity of protective lymphocytes and MIF-producing cells.

Relationship of the Protective Function of PEC and their Ability to Release MIF

PROTECTIVE ACTIVITY OF PEC. In the preceding experiments, the protective capacity and MIF-producing potential of PEC were compared on a cell-for-cell basis. But the ability of animals to focus their cellular defenses in the inflamed peritoneal cavity can be meaningfully evaluated only when cells from individual donors are transferred into individual recipients. Accordingly, exudates were induced in a large panel of 6-day *Listeria*-infected rats by stimulating the animals intraperitoneally with 10^7 killed *F. tularensis*. At predetermined intervals thereafter, groups of five rats were sacrificed and PEC obtained from them were transferred intravenously into an equal number of recipients. The cells were transfused 1 h after the recipients had been challenged intravenously with approximately 3×10^6 LM.

The results of two experiments have been combined in Fig. 3 where it can be seen that PEC harvested immediately after stimulation were devoid of protective activity. A low but significant ($P < 0.05$) level of protection was transferred by cells harvested 4–6 h after exudate induction. Thereafter, the level of adoptive immunity increased to a maximum at 24 h. The second relevant finding is that the immunity conveyed by PEC obtained from different groups of donor rats bore no obvious relationship to the number of cells transferred. This was true not only of inocula prepared from exudates of different age, but also of PEC obtained from different donors at the same interval after stimulation. For instance, Fig. 3 shows that the 24-h exudates obtained from two groups of *Listeria*-infected donors contained means of 3.1×10^7 and 6.8×10^7 cells; yet the cells transferred approximately the same level of antimicrobial resistance. This finding demon-

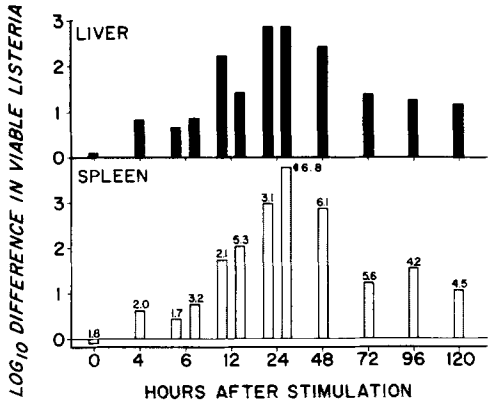

FIG. 3. Bar graphs describing the appearance in induced peritoneal exudates of cells which can protect normal recipients against a *Listeria* challenge. The donors were 6-day *Listeria*-infected rats that were stimulated intraperitoneally with killed *F. tularensis*. At the indicated intervals after stimulation, PEC from five donors were transferred intravenously into five recipients. The cells were transferred 1 h after the recipients were challenged intravenously with approximately 3×10^6 LM. The results are expressed as the mean difference 48 h after challenge in viable units in the livers and spleens of the cell recipients and a panel of five normal, nonimmunized subjects that were challenged with the same number of organisms. A significant level of immunity was conferred on the liver and spleen by cells harvested 4 h or longer after exudate induction. The mean number of cells $\times 10^7$ transferred is shown at the top of the spleen columns.

strates that the number of cells in an exudate is a fallible index of their protective power, and gives credence to the view that the arrival of sensitized lymphocytes in the inflammed peritoneal cavity can be meaningfully evaluated only on an animal-for-animal basis.

ARRIVAL OF MIF-PRODUCING CELLS IN EXUDATES. The preceding argument, developed with respect to the protective capacity of PEC, applies in principle to the cells in exudates that respond to LM antigen in vitro. But comparison of MIF production by PEC on an animal-for-animal basis entails the use of capillary tube cultures containing different ratios of test cells and indicator cells. The effect of this variation on the assay was determined in a preliminary experiment in which 24-h PEC from 6-day *Listeria*-infected donors and casein-induced indicator cells from normal rats were mixed in different proportions. In the presence of 1 μg/ml of LM antigen, similar levels of migration inhibition were observed in cell cultures containing 25–75% immune cells.

With this information, an experiment was undertaken in which the arrival of MIF-producing cells in the peritoneal cavity of *Listeria*-infected rats was measured quantitatively and kinetically over a 5-day period after induction of an exudate. In this experiment, 96 normal subjects and 96 6-day *Listeria*-infected rats were stimulated intraperitoneally with killed *F. tularensis*. At predetermined intervals thereafter, corresponding to the time points at which the protective capacity of the cells had been measured (Fig. 3), PEC from individual donors were combined in vitro with 3×10^7 indicator cells. Capillary tube cultures prepared from these mixtures were cultivated in the presence of different concentrations of LM antigen.

Fig. 4 shows that an effect of the antigen on cell migration was first detected in cultures containing immune PEC harvested 12 h after stimulation. Thereafter, responsiveness of the cells increased to a maximum at 18–36 h then declined to a level that could no longer be detected at 120 h.

Discussion

In the present investigation, quantitative methods were used to demonstrate a temporal relationship between the development of cellular hypersensitivity and cellular resistance to LM. The emergence of DTH to LM antigen was shown to coincide or closely overlap both the appearance of MIF-producing cells in inflammatory exudates and the delivery to the general circulation of lymphocytes that can protect recipient rats against a *Listeria* challenge. These findings accord with earlier studies in which a similar relationship between DTH and acquired resistance was demonstrated in animals infected with *Mycobacteria* (22),

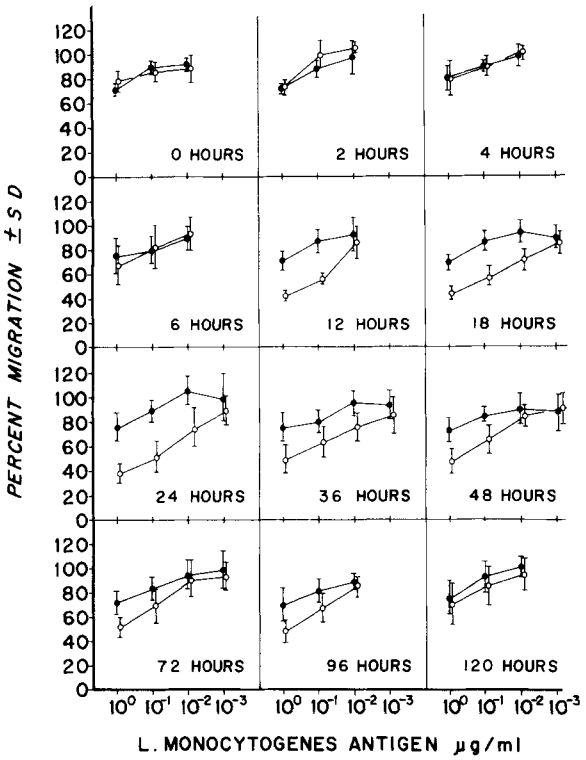

FIG. 4. Curves describing the appearance in induced peritoneal exudates of cells that are responsive to LM antigen in the MIF assay. PEC from either normal rats (●—●) or 6-day *Listeria*-infected rats (O—O) stimulated with *F. tularensis* were harvested at the indicated intervals. Cells from individual donors were combined with 3×10^7 casein-induced indicator cells. The cell mixtures were then cultivated for 24 h in the presence of different concentrations of LM antigen. The results are expressed as percent migration of the same cells cultivated in antigen-free medium. MIF production was first demonstrated by PEC harvested 12 h after stimulation. Thereafter, the activity of the cells increased to a maximum at 18–36 h then declined to an insignificant value 120 h after stimulation. Means of 5–8 ± SD.

Salmonellae (23), *Brucellae* (24), and *F. tularensis* (16, 25). Together with other evidence indicating that serum is unable to immunize passively (26), they encourage the belief that resistance to LM is cell mediated in the rat as it is in the mouse (27).

The documented association between DTH, acquired immunity, and the in vitro response of lymphocytes to microbial antigens raises questions regarding the role of soluble lymphocyte products in the expression of cellular resistance to infection. Several investigators have reported that biologically active molecules are released by a variety of lymphocytes (28–30) and even by murine fibroblast cell lines at a particular stage in the cell cycle (31). From the standpoint of cellular resistance, however, one is concerned mainly with the response of T cells and particularly with the release by specifically sensitized lymphocytes of factors that have secondary effects on macrophages. Plausible candidates in addition to MIF are the monocyte chemotactic factor (32) and substances which can enhance the metabolic and microbicidal activity of macrophages (33, 34).

To merit serious consideration, it should be possible to relate the activity of these "mediators" to processes occurring in the intact animal. The results of the present investigation are pertinent in this regard for they demonstrate that the localization of MIF-producing cells in bacteria-induced exudates parallels the arrival in the exudates of protective lymphocytes. Failure to detect specifically sensitized lymphocytes in the peritoneal cavity of infected rats at the time of exudate induction implies that both protective lymphocytes and mediator-producing cells arise elsewhere and are drawn into the peritoneal cavity in response to inflammation.

An entirely different line of evidence gives credence to this notion and points again to a mechanism whereby specifically sensitized lymphocytes are concentrated at sites of bacterial implantation. In the current study, TDL and PEC obtained from donor rats at the peak of their response to an immunizing *Listeria* infection were compared with respect to their protective activity and capacity to release MIF. On a cell-for-cell basis, PEC were approximately five times more potent. This finding takes on added significance when it is remembered that both the protective cells and MIF-producing cells are lymphocytes (14, 35, 36), and that lymphocytes accounted for less than 20% of cells in the exudates. It would therefore seem that the specifically sensitized lymphocytes in exudates are functionally more "mature" or that a mechanism exists for concentrating them in inflammatory foci. The two possibilities are not mutually exclusive—sensitized lymphocytes might localize preferentially in exudates where their subsequent differentiation enables them to interact more efficiently with macrophages.

The specifically sensitized lymphocytes which mediate cellular resistance to infection have been identified as T cells (5–7). But T cells differ with respect to size and structure, tissue disposition, and function. Indeed, T cells can carry out at least two immune functions in addition to those described—they can operate as helper cells in the antibody response (37), and can destroy target cells bearing membrane-associated antigens (38). It is pertinent to inquire, therefore, whether the ability to transfer cellular resistance and the capacity to release MIF are properties of the same cells or different cells. While this question cannot be

answered categorically, the studies reported here suggest that the cells concerned share at least one property, namely the capacity to localize in inflammatory foci.

Radiolabeling studies have shown that among the cells in central lymph, immunoblasts alone extravasate in the inflamed peritoneal cavity of recipient rats (20). It has also been demonstrated that drugs which are toxic for immunoblasts also inhibit the specifically sensitized lymphocytes which can protect recipient rats against a *Listeria* challenge (39, 40). These findings, together with the demonstrated capacity of MIF-producing cells to localize in exudates, suggest that the cells concerned emerge from the blood in a blast condition.

The local release of MIF could go far in explaining how infected animals marshal their cellular defenses at sites of microbial invasion. The basic thesis is that organisms provoke a local inflammatory response that encourages the extravasation of immunoblasts. Cells of all available specificities emigrate from the blood into the surrounding tissue where they continue to divide and differentiate. The few specifically committed lymphocytes in the exudate are stimulated by microbial antigens to release factors, such as MIF, that promote the influx, retention, and activation of monocyte-derived macrophages. It is to be emphasized that these speculations rest mainly upon observations made in in vitro systems. Nevertheless, they accord with evidence obtained in animal studies, and serve to emphasize that the expression of cellular resistance is a two-stage process—the first involving the interaction of antigen and specifically sensitized lymphocytes, and the second adaptive changes in macrophages, the nonspecific effector cells through which resistance to infection is ultimately expressed.

Summary

Acquired resistance to the intracellular bacterial parasite, *Listeria monocytogenes* can be transferred to normal recipients by thoracic duct lymphocytes or peritoneal exudate cells obtained from rats infected with this organism. The appearance of protective cells in thoracic duct lymph coincides with the development in the donors of delayed-type hypersensitivity to *Listeria* antigens and accumulation in induced peritoneal exudates of cells which are responsive to these antigens in the migration inhibitory factor (MIF) assay. The cells in exudates that confer protection, and those that release MIF, arise at sites remote from their final destination. From their point of origin in the caudal lymph nodes of infected rats, cells with these properties are delivered to the thoracic duct and hence to the blood from where they are drawn into the peritoneal cavity in response to inflammation. The parallel observed in the appearance, increase and subsequent decline of protective lymphocytes and MIF-producing cells in exudates suggest that the two activities are mediated by a single line of T cells. However this may be, the development and deployment of the cells concerned encourages the belief that MIF has a meaningful role in the expression of cellular resistance to infection.

The assistance of Ms. Janet Kubli is gratefully acknowledged.

Received for publication 10 February 1975.

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