THE EFFECT OF CYTOTOXIC AGENTS ON THE PASSIVE TRANSFER OF CELL-MEDIATED IMMUNITY*

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It is generally accepted that acquired resistance against intracellular bacterial parasites rests with the macrophage. A series of investigations from this laboratory has shown that the specific immune mechanism which leads to activation of macrophages, and consequent immunity, is mediated by cells belonging to the lymphoid series (1, 2). The passive transfer of specifically immune lymphoid cells to normal recipients was shown to confer immediate protection against challenge with *Listeria monocytogenes*. Obviously, some mechanism must exist for an interaction between the donor lymphoid cells and the host macrophages.

Although the nature of the underlying mechanism in acquired cellular resistance is still obscure, the adoptively immunized animal lends itself to an illuminating extension of the observations reported in a companion paper (3). Here it was shown that the administration of various cytotoxic drugs effectively suppresses the development of acquired resistance in *Listeria*-infected mice. But it could not be concluded unequivocally that the effects observed were always due to interference with the host's immune response, because the drugs examined have broadly cytotoxic properties, and were used in high dosage. This uncertainty was overcome in the present report by using passively immunized animals to distinguish between specific interference with the immunological components of the host's cellular defenses and spurious effects which do not result from immunosuppression in its accepted sense.

Materials and Methods

Mice.—Female specific pathogen-free (SPF) mice, aged 5–7 wk and weighing between 18 and 22 g, were used.

Organisms.—Listeria monocytogenes, strain EGD, was used. This strain has been maintained by alternate passage in the mouse spleen and trypticase soy broth (TSB) medium, and has a LD_{50} of approximately 5×10^8 viable *Listeria*.

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Infection.—Mice were infected with a logarithmic phase culture of *L. monocytogenes* in TSB medium, appropriatedly diluted with Hanks' balanced salt solution to contain the desired number of bacteria in 0.2 ml of the inoculum. Inocula of between $10^3 - 5 \times 10^3$ viable *Listeria* were used for immunization of prospective donors. The dose used to challenge passively immunized recipients varied between 10^4 and 10^5.

Preparation and Transfer of Cell Suspensions.—The procedure for preparing spleen cell suspensions has been described in detail elsewhere (1). The donor mice were killed on day 7 of the immunizing infection and were used to prepare filtered spleen cell suspensions which were injected intravenously into recipients in a dose of approximately 2×10^8 cells (viability 80 – 90%) in a volume of 0.5 ml. The general design consisted of injecting 10 recipients for each experimental group under study.

Drugs.—AZT[1] (Imuran, generously supplied by Burroughs Wellcome & Co., Tuckahoe, N. Y.). MTX[1] (Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N. Y.) and CY[1] (Cytoxan, Mead Johnson & Co., Evansville, Ind.) were injected as aqueous solutions subcutaneously in doses of 200, 50, and 200 mg/kg body-weight, respectively. VLB[1] (vincaleucoblastine sulfate, Velban, Eli Lilly and Co., Indianapolis, Ind.) was administered intravenously in a dose of 4 mg/kg. All drugs were given as single dose treatments, administered on the day specified. These dosages have been shown to be effective in suppressing the primary immune response to *Listeria* infection in mice (3).

Irradiation.—In the experiments involving irradiation, groups of prospective recipient mice were irradiated with 900 rads X-irradiation 24 hr prior to passive immunization with immune lymphoid cells.

Measurement of Protective Immunity.—Mice were challenged with an intravenous injection of approximately 10^4 viable *Listeria immediately after cell transfer.* In each challenge experiment, 15 normal mice were included as controls; five of these were sacrificed at 10 min after infection in order to establish the bacterial content in the liver and the spleen at the start of infection; thereafter, bacterial counts were made on five mice from each group at 24 and 48 hr. The viable counts were determined by plating appropriate dilutions of homogenates of liver and spleen on TSA medium and incubating the plates overnight at 37°C.

RESULTS

Protective Immunity in Passively Immunized Mice.—

A preliminary study was undertaken to assess the degree of protection provided by the passive transfer of immune lymphoid cells to normal recipients. The course of challenge infection was studied in three groups of mice: (*a*) the immune donors challenged on the day of transfer; (*b*) a group of passively immunized mice which received immune lymphoid cells from 7-day immune donors; and (*c*) normal controls. The results are presented in Fig. 1.

Listeria multiplied in the livers and spleens of the control mice during the first 48 hr of infection. In contrast, the actively immunized donor mice were highly resistant to the challenge infection; a progressive fall occurred in the viable count during 48 hr. Animals which received immune lymphoid cells showed a substantial degree of protection which was more rapidly established in the spleen than in the liver.

Protective Immunity in Mice Treated with a Single Dose of Drug on the Day of Passive Immunization.—

Three groups of mice were used to study the effect of cyclophosphamide on the passive transfer of immunity: (*a*) a group of passively immunized mice treated with a single dose of

[1] AZT, azathioprine; MTX, methotrexate; CY, cyclophosphamide; VLB, vinblastine.

cyclophosphamide at the time of cell transfer; (*b*) a group of passively immunized controls; and (*c*) a group of unimmunized controls. The course of infection was followed for 48 hr.

The results, presented in Fig. 2, showed that *Listeria* underwent rapid multiplication in control mice during 48 hr. Passively immunized mice showed considerable immunity, as indicated by the lower viable counts in spleens and livers at 24 and 48 hr. The *Listeria* counts in the cyclophosphamide-treated

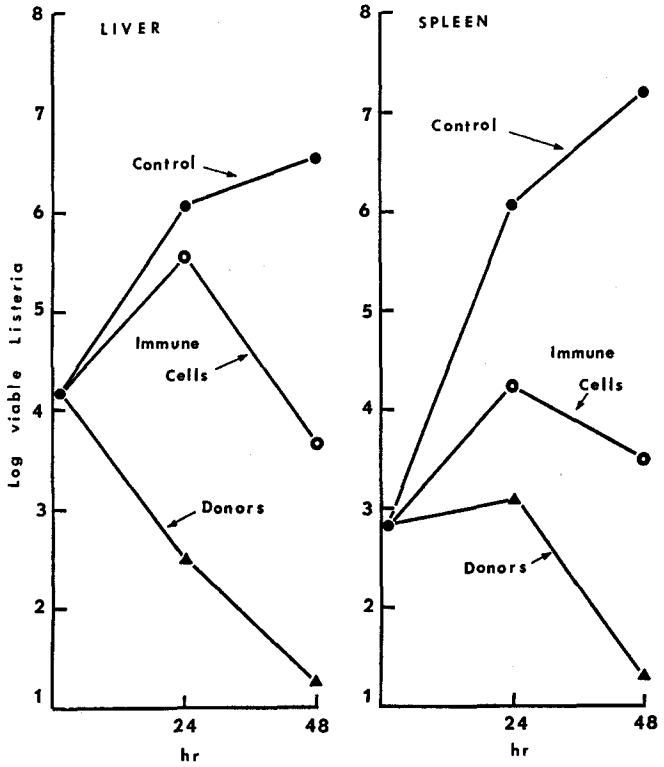

FIG. 1. Growth curves of *L. monocytogenes* in 7-day immune donors, the recipients of 2 × 10^8 immune lymphoid cells, and in normal controls. Means of 5.

recipients of immune cells were considerably higher than in the corresponding untreated animals, indicating the suppression of passive transfer of immunity by cyclophosphamide.

Experiments of similar design were used to study the effects of vinblastine, methotrexate, and azathioprine. The results are presented in Figs. 3–5. In each of the experiments, passively immunized mice exhibited immunity to *Listeria* challenge which was evident at 24 and at 48 hr in the spleen, but usually only at 48 hr in the liver (1).

Vinblastine administered to passively immunized mice at the time of in-

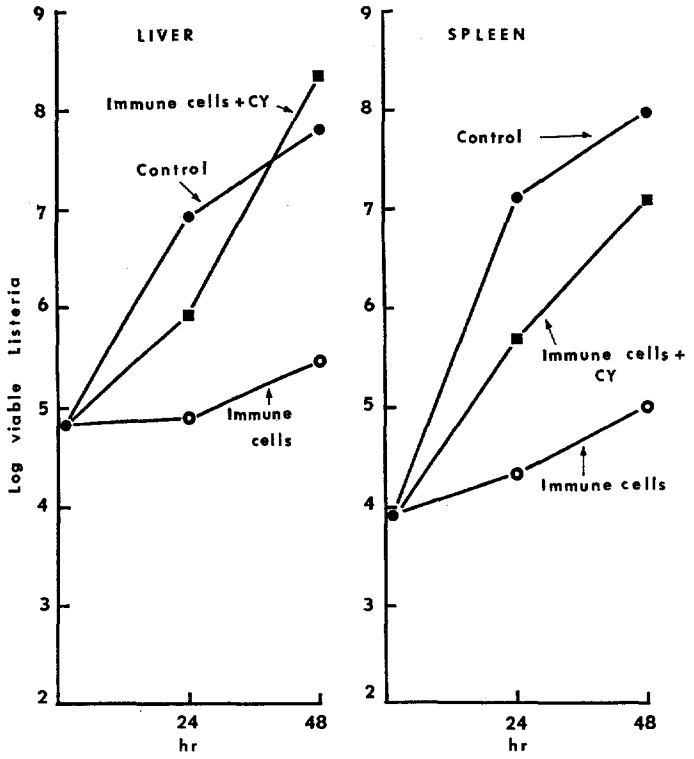

FIG. 2. Growth curves of *L. monocytogenes* in normal controls and two groups of recipients of immune spleen cells to one of which cyclophosphamide (CY) was administered at the time of cell transfer.

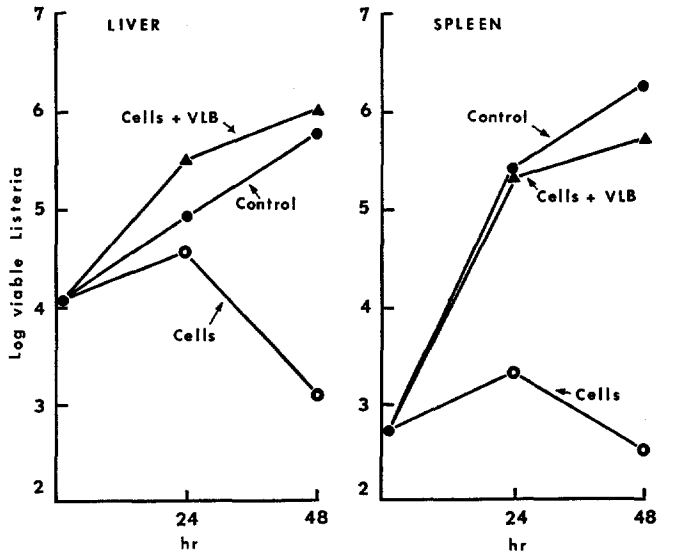

FIG. 3. Growth curves of *L. monocytogenes* in normal controls and two groups of recipients of immune spleen cells to one of which vinblastine (VLB) was administered at the time of cell transfer.

fection effectively suppressed the passive transfer of immunity (Fig. 3). Methotrexate, on the other hand, had only a slight effect (Fig 4), while azathioprine had no effect at all (Fig. 5).

Effect of Treatment of Recipients with Single Doses of Drugs a Day Prior to Passive Immunization.—In the preceding section, treatment of recipients with cyclophosphamide or vinblastine at the time of cell transfer was found to

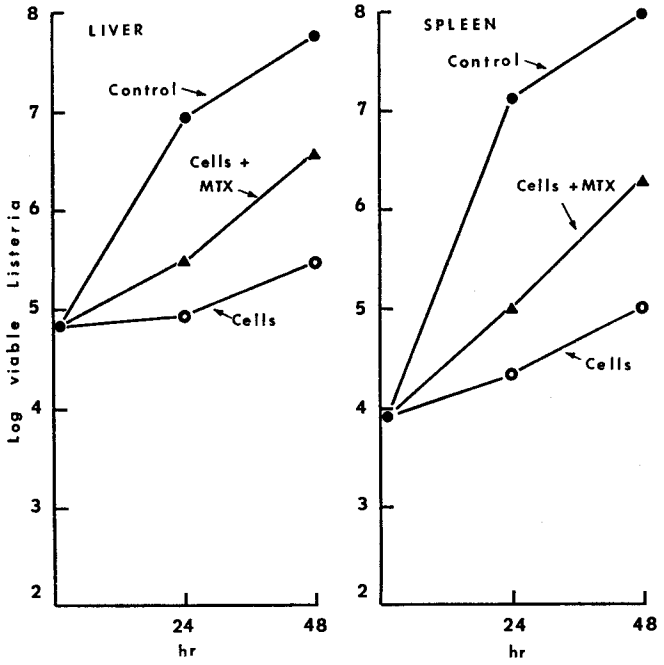

FIG. 4. Growth curves of *L. monocytogenes* in normal controls and two groups of recipients of immune spleen cells to one of which methotrexate (MTX) was administered at the time of cell transfer.

suppress passive immunization. It was not clear, however, whether the immunosuppressive activity was the result of a cytotoxic action on the transferred immune lymphoid cells or on recipient cells which may also be required for the expression of passive immunity.

To elucidate this problem, prospective recipients were treated with single injections of cyclophosphamide or vinblastine 24 hr before the scheduled time of cell transfer; the course of *Listeria* infection in these mice, when challenged at the time of cell transfer, was studied in relation to the growth curves in control mice and in passively immunized mice which had received no drug. The results are presented in Fig. 6 for cyclophosphamide, and in Fig. 7 for vinblastine.

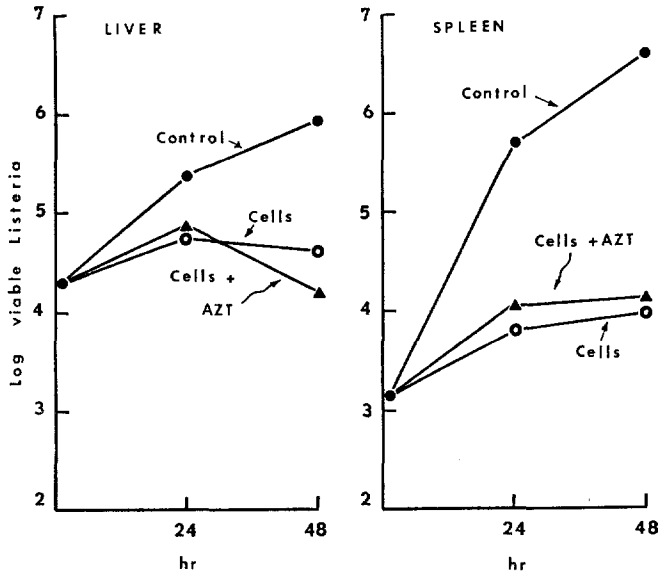

FIG. 5. Growth curves of *L. monocytogenes* in normal controls and two groups of recipients of immune spleen cells to one of which azathioprine (AZT) was administered at the time of cell transfer.

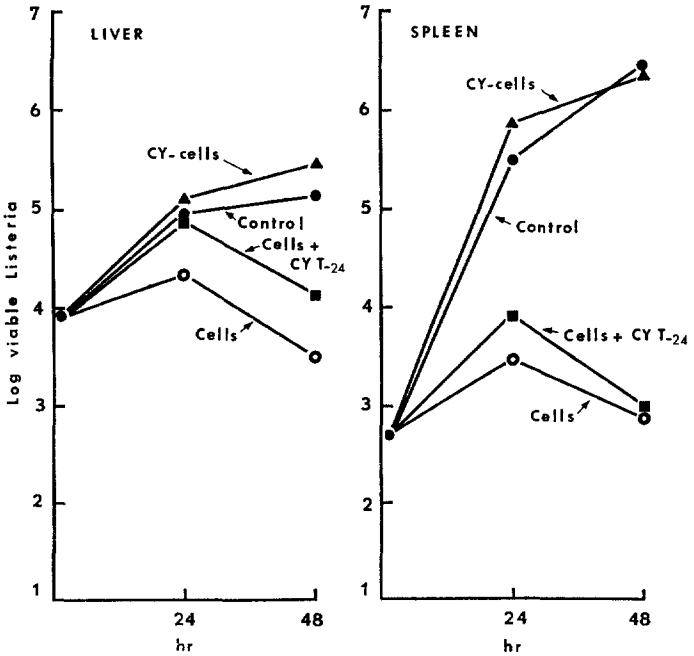

FIG. 6. Growth curves of *L. monocytogenes* in: normal mice (control); recipients of immune cells from cyclophosphamide-treated donors (CY-cells); recipients of immune cells from untreated immune donors (cells); and recipients which had been treated with cyclophosphamide 24 hr prior to transfer of immune spleen cells (cells + CY T_{-24}).

Prior treatment of recipients with cyclophosphamide or vinblastine failed to prevent the transfer of immunity by immune lymphoid cells; this suggests that immunosuppression resulting from the administration of these drugs to recipients at the time of cell transfer is due to their action on the transferred cells.

Effect of Treatment of Donors with Single Doses of Drugs a Day Prior to Harvesting of cells.—

The effect of cyclophosphamide on the immunologically committed cells of the donors was studied by treating half of a group of immunized mice with a single injection of cyclophospha-

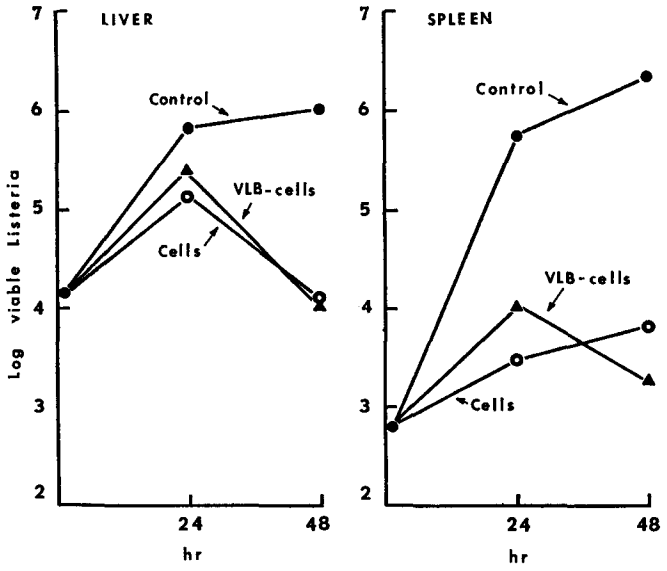

FIG. 7. Growth curves of *L. monocytogenes* in normal mice (control), in recipients of spleen cells from immune donors (cells), and immune donors which had been treated with vinblastine 24 hr before harvest (VLB-cells).

mide on the 6th day of the immunizing infection. The protective capacity of the lymphoid cells from these animals obtained a day later was compared with that of the cells from untreated donors.

The results (Fig. 6) showed that while cells from untreated donors were protective, an equal number of viable cells from cyclophosphamide-treated animals were not. Thus, the immune lymphoid cells were sensitive to the cytotoxic effect of cyclophosphamide.

A similar experiment with vinblastine showed that treatment of donors with vinblastine 24 hr before harvesting of cells did not affect their functional activity in recipients (Fig. 8). This suggests that the immune lymphoid cells

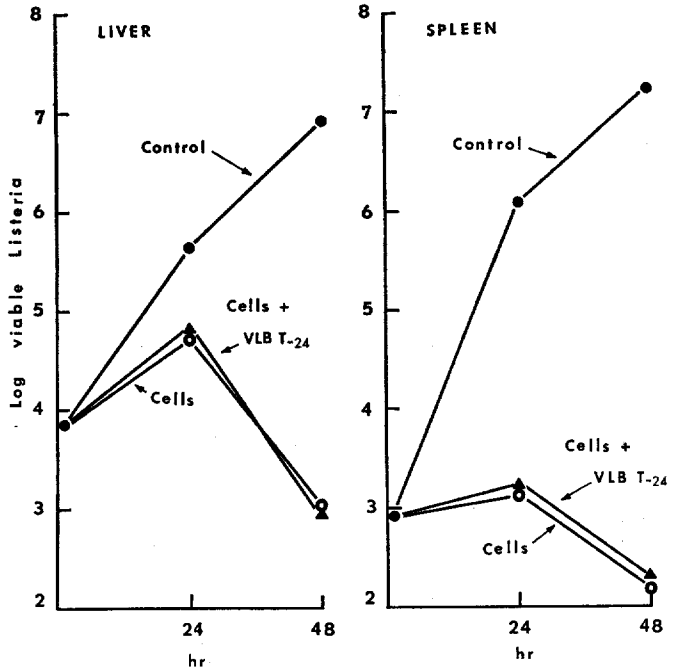

FIG. 8. Growth curves of *L. monocytogenes* in: normal mice (control); recipients of immune spleen cells (cells); and recipients which had been treated with vinblastine 24 hr prior to transfer of immune spleen cells (cells + VLB T_{-24}).

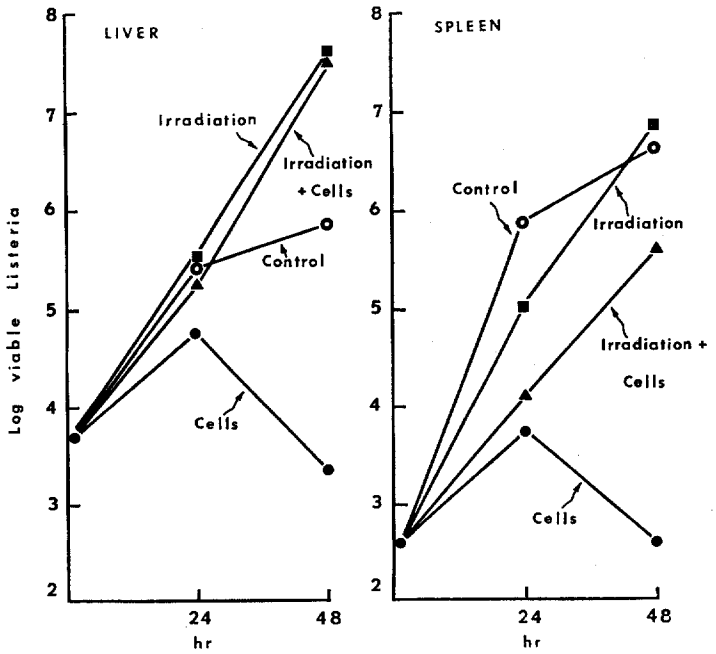

FIG. 9. Growth curves of *L. monocytogenes* in normal or irradiated controls and in normal or irradiated recipients of immune spleen cells.

were in a resting phase and hence insensitive to the action of vinblastine which is a mitotic poison.

Effect of Irradiation of Recipients on Passive Immunization.—The experiments on pretreatment of recipients with cyclophosphamide reported in the preceding section indicated that the host component required for the mediation of passive immunization was insensitive to the action of this drug. Since cyclophosphamide is known to be toxic to the lymphoid series of cells (4) and nontoxic to macrophages,[2] it was reasonable to assume that macrophages and their precursors may be the host components required for the mediation of passive immunization. The following experiments were undertaken to elucidate this question.

A group of prospective recipients was exposed to 900 rads X-irradiation. After 24 hr they were injected with specifically immune lymphoid cells and challenged with *Listeria*; the course of the *Listeria* infection is presented in Fig. 9 where it is compared with that found in unimmunized hosts and in passively immunized animals which had not been irradiated.

The results indicated that prior irradiation of recipients abolished their capacity to benefit from passive immunization, suggesting that protection depends upon the intervention of a radiosensitive cell supplied by the recipient. The growth curve shows, however, that partial protection existed for the first 24 hr after transfer; thereafter, a steep, seemingly logarithmic rise occurred in the *Listeria* count. This suggests that the recipient was not able to sustain its contribution to the immune mechanism. The most plausible explanation is that the supply of phagocytic cells became exhausted. The studies of Volkman and Gowans (5, 6), and Volkman and Collins (7) have shown that blood monocytes arise from radiosensitive precursors in bone marrow. This cell type virtually disappears from circulation within 24 hr of irradiation. The tissue macrophage which derives from blood monocytes is thus the cell most likely to be missing from the defense mechanism of irradiated recipients.

DISCUSSION

Cytotoxic agents were employed in the present investigation with a view to elucidating the cellular events which underlie the acquisition of antimicrobial resistance by animals which have been passively protected with immune lymphoid cells. Recipients were injected intravenously with 2×10^8 spleen cells obtained on day 7 of an immunizing infection with *L. monocytogenes*. These were the conditions which had been found to provide optimal protection against *Listeria* challenge (1). Four drugs and X-irradiation, representing five broad categories of cytostatic agents, were studied.

There is persuasive evidence that the primary immune response to *Listeria*

[2] Mackaness, G. B., and S. P. Tripathy. 1969. The effect of cytotoxic agents on established resistance to *Listeria monocytogenes*. Manuscript in preparation.

infection is generated through the specific induction of cells belonging to the lymphoid series. Bacterial inactivation, however, is not accomplished by these cells; it is ultimately expressed nonspecifically through macrophages. Immunity in *passively* immunized animals is also specific in induction, but is again manifested nonspecifically through macrophages. The latter conclusion is based upon the finding that macrophages with nonspecific microbicidal properties and inincreased spreading activity, characteristic features of activated macrophages, appear in the peritoneal cavity of recipients which have been protected with immune lymphoid cells and challenged simultaneously with the homologous organism. The donor cells which have this capacity to confer immunity on normal recipients belong to the lymphoid series (1, 2). The exact sequence of events leading to the eventual activation of host macrophages following transfer of donor immune lymphoid cells is not known; some information, however, is obtained from the results of the present study.

Cyclophosphamide is known to be toxic to all cells belonging to the lymphoid series (4); it does not, however, affect the functional capacity of the macrophage.[1] Treatment of recipients with cyclophosphamide would therefore result in depletion of lymphoid tissue without impairment of macrophage activity. The failure of pretreatment of recipients with cyclophosphamide to interfere with the passive transfer of immunity (Fig. 6) suggests that participation of recipient lymphoid cells is not a prerequisite for the activation of recipient macrophages. The total ablation of passive immunity with cyclophosphamide administered at the time of cell transfer is probably due, therefore, to its direct lethal action on the donor lymphoid cells. This view is supported by the failure of what viable lymphoid spleen cells could be obtained from cyclophosphamide-treated donors to confer protection upon normal recipients (Fig. 6).

Vinblastine is a mitotic poison which selectively acts on cells entering mitosis, causing metaphase arrest and subsequent death (8); its action is directly proportional to the level of cellular proliferation (9). Injection of vinblastine to prospective donors on day 6 of an immunizing infection failed to affect the immunologic capability of cells obtained on day 7; this observation, considered in conjunction with the finding that vinblastine failed to influence host resistance when injected on day 7 of a primary infection (3), suggests that stabilization of the immune cell population had occurred by this time, and that the end-product of the immune response (the immunologically committed lymphoid cell) has entered a resting or intermitotic state by the 6th day of the immune response.

In contrast to its inertness when given to donors, vinblastine administered to recipients at the time of cell transfer completely suppressed the development of resistance. This implies that the acquisition of resistance involves multiplication of donor lymphoid cells or recipient phagocytes, or possibly both cell types. In any case, vinblastine must cause the virtual elimination of one of the compo-

nents necessary to the acquisition of protective immunity in passively immunized recipients. The following observations provide support for the contention that both donor lymphoid cells and recipient phagocytes do engage in multiplication as part of the host's defense mechanism.

Earlier experiments (1) on the effect of mitomycin C indicate that this drug interferes to some extent with the immunologic capability of donor lymphoid cells. It was found that immune cells which had been treated with this drug in vitro conferred a low but persisting level of protection that was unlike the steeply mounting immunity found in recipients of untreated cells. Since mitomycin C at a low concentration is known to prevent multiplication of cells without impairment of protein synthesis (10), it would seem that optimal protection with immune lymphoid cells depends upon donor cell proliferation in the recipient (1).

The fact that prior irradiation of recipients prevents them from acquiring the resistance normally conferred with immune lymphoid cells suggests that a radiosensitive component of the defense mechanism is contributed by the recipient itself. Although macrophages are not directly affected by irradiation (11), recruitment of this cell type is known to require an intact mechanism for the production of circulating monocytes (5). The monocyte precursors in bone marrow are continuously dividing cells which are extremely radiosensitive (6). Since the normal monocyte has a life span in the circulation of only 1 day, irradiation results in a rapid depletion of circulating monocytes (7). Hence, the present finding that irradiated mice show some evidence of immunity during the first 24 hr after cell transfer but none thereafter, suggests that the recruitment of blood monocytes is essential to the defenses of passively immunized recipients.

From the effect of mitomycin C on donor cells and of prior X-irradiation on recipients, it is evident that protective immunity depends upon cellular proliferation affecting both donor cell and host cell components of the defense. Vinblastine, therefore, could exert its influence through both of these cell populations. The immediate immunosuppressive effect of vinblastine in passively immunized animals, in contrast to what was seen following irradiation, suggests that its activity is largely due to its effect on the donor cells.

In the present experiments, methotrexate was only partially immunosuppressive in its effect on the passively immunized animal, and was seen in the previous paper to cause only transient suppression of the immune response in actively immunized animals (3). It is possible therefore that in both situations, a proportion of the immunologically committed lymphocytes may have escaped the thymidineless death produced with methotrexate (12, 13). Apparently those which survive go on to provide a low level of protection for the recipient.

The position is clearly different in the case of mitomycin C or vinblastine. Mitomycin C can prevent multiplication of cells without interfering with pro-

tein synthesis (10) so that the immunity conferred with mitomycin C–treated cells, as reported in another paper (1), presumably results from the activities of the cells transferred, unaided by any augmentation through cellular proliferation. This would explain the low but persisting level of protection conferred with cells which have been treated with this drug. This evidence argues convincingly that donor lymphoid cells proliferate in recipients, presumably as a result of specific antigenic stimulation. The findings with vinblastine tend to confirm this view since this drug, when administered to recipients at the time of cell transfer, totally abolished the protective capacity of the immune lymphoid cells.

The failure of azathioprine to interfere with passive immunization suggests that it may not be active against immunologically committed cells. It did, however, interfere with the development of immunity during a primary *Listeria* infection (3), suggesting that its immunosuppressive effect may be limited to a step in the inductive phase of the immune response, as has been suggested in the case of a closely allied drug, 6-mercaptopurine (14).

A feature of interest was the finding that the three drugs, cyclophosphamide, vinblastine, and methotrexate, which suppressed the passive transfer of cellular immunity, were also effective in suppressing the primary immune response to *Listeria* (3). It would thus seem that while drugs which suppress the passive transfer of immunity also suppress the primary response, the converse may not be true, since azathioprine failed to influence resistance in passively immunized mice. Suppression of resistance in passively immunized animals must result therefore from a direct action of drugs on the immunologically committed lymphoid cell. This view seems tenable since prior treatment of recipients with cyclophosphamide or vinblastine did not prevent the passive transfer of immunity with immune lymphoid cells.

No experiments have been performed as yet to study the vulnerability of the immunologically committed cell to X-irradiation. It is possible, despite the radioresistance of an ongoing antibody response, that they would prove to be susceptible to ionizing irradiation in the system studied. It is even more likely that X-irradiation should be effective in suppressing the primary immune response to *Listeria* through its destructive action on uncommitted lymphoid cells. That this is indeed so is suggested from the shapes of the growth curves in the irradiated and unirradiated controls (Fig. 9); while an immune response was evident at 48 hr in the liver of normal mice, bacterial counts were considerably higher in the irradiated controls, suggesting an immunosuppression of the primary response by X-irradiation.

The findings in the present study indicate that the specific immunologically committed lymphoid cell responsible for mediating the passive transfer of acquired cellular resistance is a resting cell which is sensitive to cyclophosphamide, is partially sensitive to methotrexate, but is insensitive to azathioprine, and also to vinblastine while it remains in the resting state.

SUMMARY

A system involving the passive transfer of committed lymphoid cells from *Listeria*-immune donors has been used to study the phases of the immune response which are sensitive to the immunosuppressive action of various cytotoxic agents. The agents investigated included cyclophosphamide, vinblastine, methotrexate, azathioprine, and X-irradiation.

Complete suppression of passive immunization was obtained by the administration of cyclophosphamide or vinblastine to recipients at the time of cell transfer or by prior X-irradiation of recipients a day before cell transfer. Methotrexate was only partially suppressive, whereas azathioprine had no effect at all. The donor cell responsible for the transfer of immunity to recipients was shown to be a resting cell which is sensitive to the action of cyclophosphamide but not to vinblastine.

The results of this investigation suggest that the donor cells undergo multiplication in the tissues of the recipient, presumably in response to specific stimulation by *Listeria* antigens. This in turn results in the activation of host macrophages. The immunosuppressive action of cyclophosphamide, vinblastine, and irradiation in the cell-transfer system has been discussed in relation to a direct cytotoxic action on the immune lymphoid cells of the donor and specific interference with their proliferation in the recipient, as well as impairment of macrophage production on the part of the recipient itself.

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