THE EFFECT OF CYTOTOXIC AGENTS ON THE PRIMARY IMMUNE RESPONSE TO LISTERIA MONOCYTOGENES*

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(Received for publication 20 December 1968)

Drugs used in the chemotherapy of cancer have also been used as immunosuppressive agents for the control of immune mechanisms involving antibody production, and cell-mediated forms of immunity such as delayed-type hypersensitivity and transplantation immunity (1–5); there are, however, few published reports of their effect on the immune response to microorganisms despite infection being a serious complication of their use in clinical practice. The present report deals with the influence of some immunosuppressive drugs on the immune response to a facultative intracellular parasite.

Tuberculosis, brucellosis, listeriosis, and salmonellosis are examples of infections which give rise to a cell-mediated form of immunity which evolves through similar mechanisms (6); it is therefore logical to assume that observations on one host-parasite system will have relevance to the others. Listeriosis in the mouse has been chosen as a model for the present study because it offers several advantages. Firstly, the immune response sets in early and runs a short course, so that an immunosuppressive effect is quickly revealed; secondly, a *Listeria* infection, as assessed by bacterial enumeration, provides an accurate and quantitative assessment of immunity and the magnitude of the immunosuppressive effect obtained with drugs. Mice infected with a sublethal dose of *Listeria monocytogenes* develop an immune response which interrupts the growth of the organism in vivo. The experiments to be described are based upon the premise that effective suppression of the immune response by drugs would result in continued multiplication of *Listeria*, particularly in the spleen and liver.

Four drugs, representing four different categories of cytotoxic agents, were chosen for study: (*a*) cyclophosphamide, a polyfunctional alkylating agent; (*b*) vinblastine, an antimitotic agent; (*c*) methotrexate, a folic acid antagonist; and (*d*) azathioprine, a purine analogue. The present report deals with the effect of these four drugs on the immune response to *Listeria* infection in mice.

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* These investigations were supported by Grant AI-07015 from the National Institute of Allergy and Infectious Diseases, and by a grant from the National Tuberculosis and Respiratory Disease Association.

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Materials and Methods

Mice.—Female specific pathogen-free (SPF) mice derived from COBS progenitors were used. The mice were about 5–7 wk old and usually weighed between 18 and 22 g at the time of infection.

Infecting Strain.—Listeria monocytogenes, strain EGD, was used. This strain has been maintained in this laboratory by alternate passage in the mouse spleen and trypticase soy broth (TSB) medium. It has remained in the smooth phase and has an intravenous LD_{50} of approximately 5×10^3 viable units. Growth was unaffected by 100 μg/ml of cyclophosphamide, vinblastine, methotrexate, or azathioprine when the strain was exposed to these drugs in TSB medium in vitro.

Infection.—The bacterial content in a logarithmic phase culture of *L. monocytogenes* was determined by direct count (Petroff-Hausser). It was appropriately diluted in Hanks' Balanced Salt Solution to contain the desired number of bacteria in 0.2 ml, the volume used to infect animals by intraveous injection. A viable count of the inoculum suspension was invariably made by plating appropriate dilutions of the suspension on trypticase soy agar (TSA) medium.

Bacterial Enumeration in Liver and Spleen.—Autopsies were conducted on five mice from each group at each time-point. Five mice were killed 10 min after injection to establish the initial bacterial content in spleens and livers; thereafter bacterial counts were made at intervals on five mice from each group at each time-point.

The mice were killed by cervical dislocation. Organs were removed aseptically to homogenizer tubes containing 8 ml (for liver) or 9 ml (for spleen) of TSB medium, giving final volumes of approximately 10 ml. They were dispersed for 1 min with a Teflon homogenizer. Appropriate serial dilutions of each homogenate were then plated on TSA medium. The viable bacterial content of the viscera was estimated from colony counts after overnight incubation at 37°C. The geometric means of the viable counts of *Listeria* in the livers and spleens were calculated separately for each group. All the results have been presented in this report as mean viable counts expressed on a logarithmic scale.

Drug Treatment.—MTX[1] (Lederle Laboratories, Division of American Cyanamid Co., Pearl River N. Y.), CY[1] (Cytoxan, Mead Johnson & Co., Evansville, Ind., and AZT[1] (Imuran, generously supplied by Burroughs Wellcome & Co., Tuckahoe, N. Y.) were administered subcutaneously as aqueous solutions in 0.25 ml containing doses equivalent to 50, 200, 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, contained in 0.1 ml. Unless otherwise specified, all drugs were given as single dose treatments, administered on the day specified. The dosages selected represent the maximum tolerated dose for each drug, based on observations of Berenbaum and Brown (7) for cyclophosphamide and vinblastine and our own unpublished observations on all four drugs.

RESULTS

Listeria Growth Pattern in Normal Mice.—

A preliminary study was undertaken to determine the most suitable infecting dose of *Listeria monocytogenes*. Four groups of mice were infected with varying doses of *L. monocytogenes*. Viable counts were performed at frequent intervals thereafter, as recorded in Fig. 1.

The course of infection in the liver was similar in all four groups during the first 24 hr. Beyond that time, the shape of the growth curve was dependent upon

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

the infecting dose. All groups showed an initial fall, ranging from 30–81% at 3 hr; thereafter, there was a steady and parallel rise during the next 21 hr. Between 24 and 48 hr, bacterial growth continued unchecked only in the animals infected with the largest dose of organisms; all others showed an inflection which was interpreted as evidence of an immune response on the part of the host tending to limit further multiplication of the organisms.

The spleen counts showed no evidence of an initial fall; growth continued log-linearly in the spleen for only 12 hr. Thereafter, there was a change in the

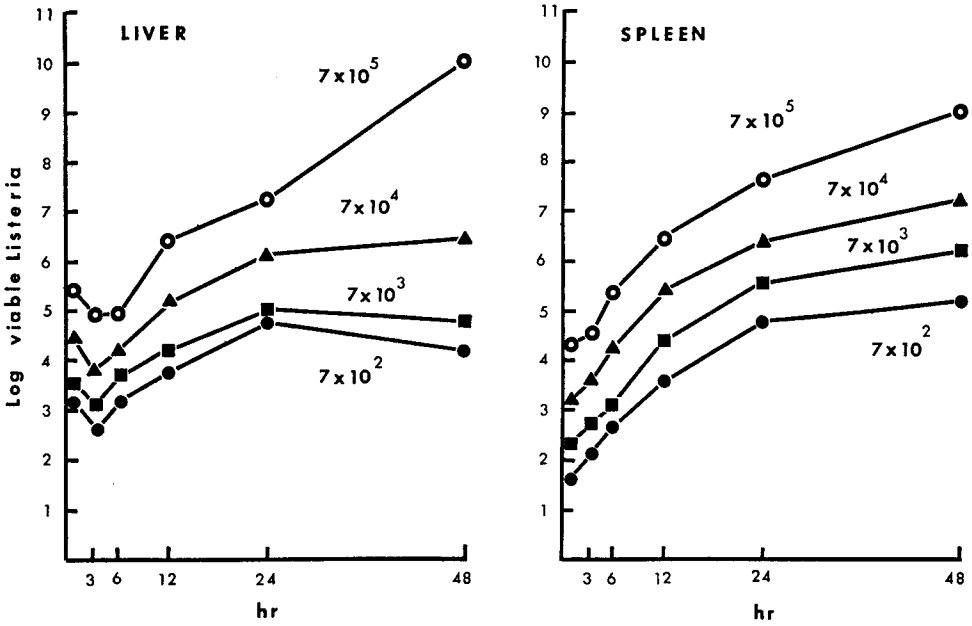

FIG. 1. Growth curves of *L. monocytogenes* in livers and spleens of mice infected at varying dose levels. Each plot represents the mean from five mice.

slope, most marked at lower dose levels. Although the growth curve indicates some inhibition with the largest infecting dose, the effect is spurious since it results from the inability of a necrotic spleen to support a bacterial population much larger than 10^8 *Listeria*.

It is apparent that the effect of developing resistance is most obvious in both liver and spleen with a low infecting dose of organisms. For this reason the effect of immunosuppressive drugs was studied in animals infected with less than 10^4 bacteria.

The Course of Infection in Mice Treated with a Single Dose of Drug on the Day of Infection.—

Three groups of mice infected with 2×10^3 *Listeria* were treated with single injections of cyclophosphamide, vinblastine, and methotrexate; the course of infection in the three groups

was plotted daily, using five mice from each group at each time-point. The growth curves were compared with those in control mice which received no treatment. The results are recorded in Fig. 2.

The liver counts indicated that untreated mice exhibited an immune response resulting in effective control of bacterial multiplication beyond 24 hr. In

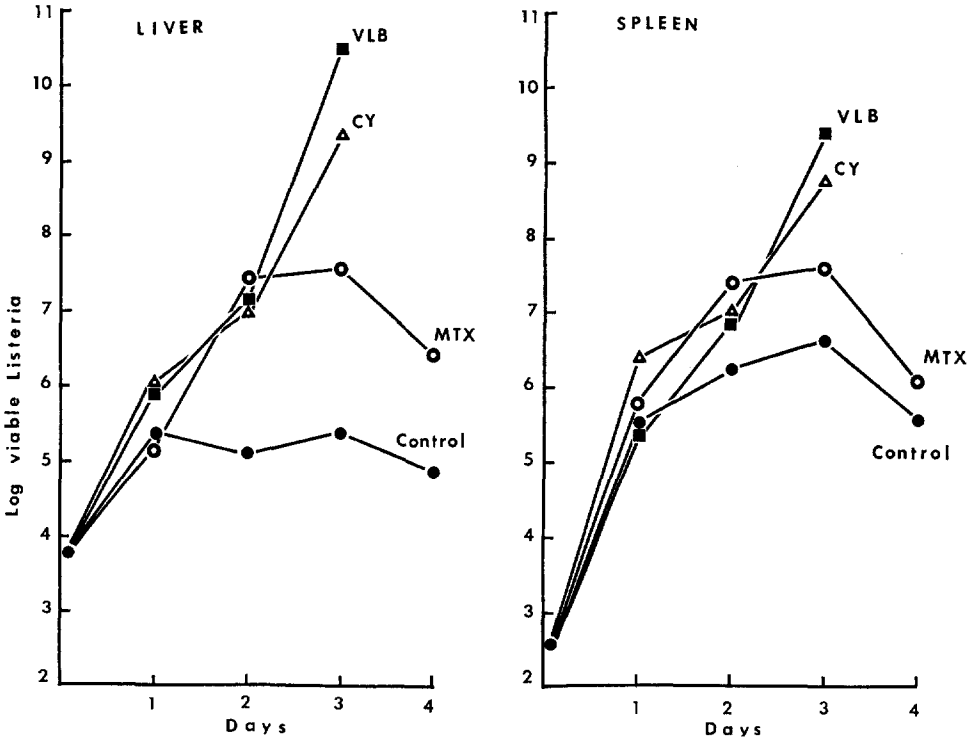

FIG. 2. Growth curves of *L. monocytogenes* in the livers and spleens of untreated mice (control) and mice given a single dose of cyclophosphamide (CY), vinblastine (VLB), or methotrexate (MTX) on the day of infection. All remaining mice in groups VLB and CY died between days 3 and 4.

contrast, the immune response was effectively suppressed in the three drug-treated groups, as evidenced by continued bacterial growth. In comparison with the controls, bacterial counts at 48 hr were significantly higher for cyclophosphamide ($P < 0.05$), vinblastine ($P < 0.01$), and methotrexate ($P < 0.05$). The bacterial content continued to increase between 48 and 72 hr in cyclophosphamide- and vinblastine-treated mice, resulting in the death of all remaining mice between 3 and 4 days.

The effects of methotrexate were markedly different from those of cyclo-

phosphamide and vinblastine. Its immunosuppressive effect persisted for only 48 hr, as is evidenced by the shape of the curve beyond that time. This suggests that the immunosuppressive effect of methotrexate, in contrast to that of cyclophosphamide or vinblastine, is reversible.

The course of infection in the spleen presented broadly similar features except that the magnitude of the differences between the bacterial populations in the drug-treated and the control groups was smaller.

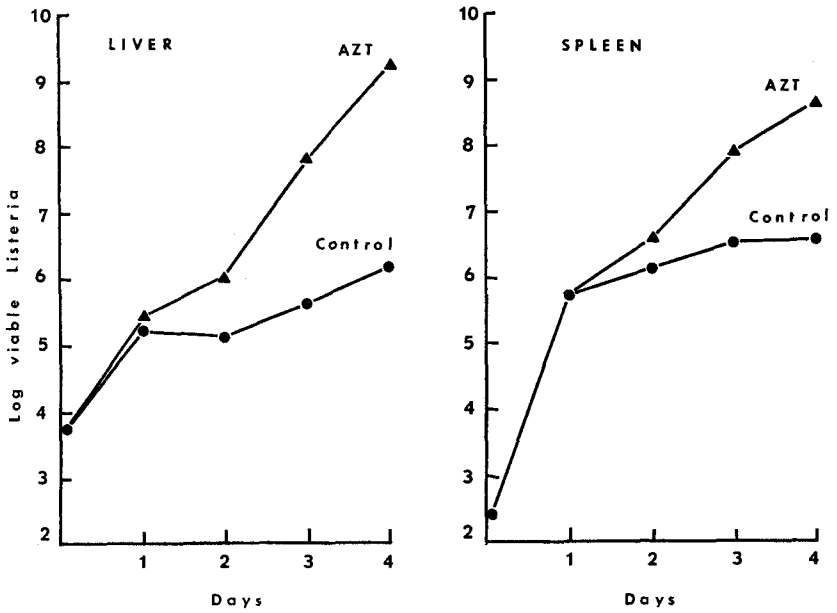

FIG. 3. Growth curves of *L. monocytogenes* in control mice and mice treated with a single injection of azathioprine (AZT) at the time of infection.

In a similar experiment, the influence of azathioprine was studied. The results, presented in Fig. 3, showed that azathioprine completely suppressed the immune response; the differences between the control and the drug-treated groups at 72 and 96 hr attained significance ($P < 0.05$ and < 0.01, respectively).

Effect of Repeated Administration of Methotrexate on the Course of Infection.— The transient nature of the immunosuppression achieved with a single injection of methotrexate on the day of infection prompted a study to determine whether an immunosuppressive effect could be sustained by the administration of a second injection of MTX at 24 hr. The results, presented in Fig. 4, showed that complete suppression of immunity was achieved only in animals which received a second injection of methotrexate.

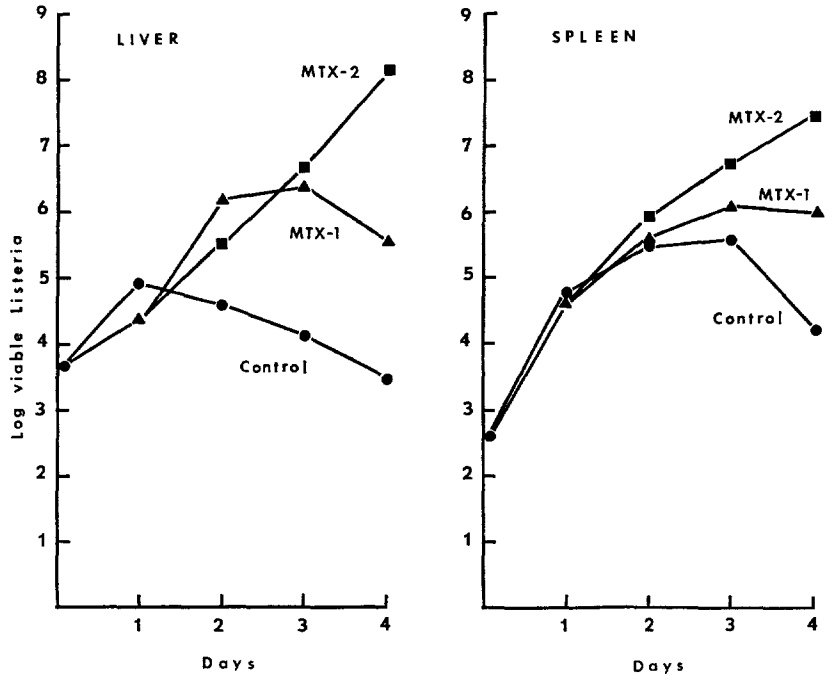

FIG. 4. Growth curves of *L. monocytogenes* showing the effects of a single dose of methotrexate given at the time of infection (MTX-1); and of two doses given at infection and 24 hr later (MTX-2).

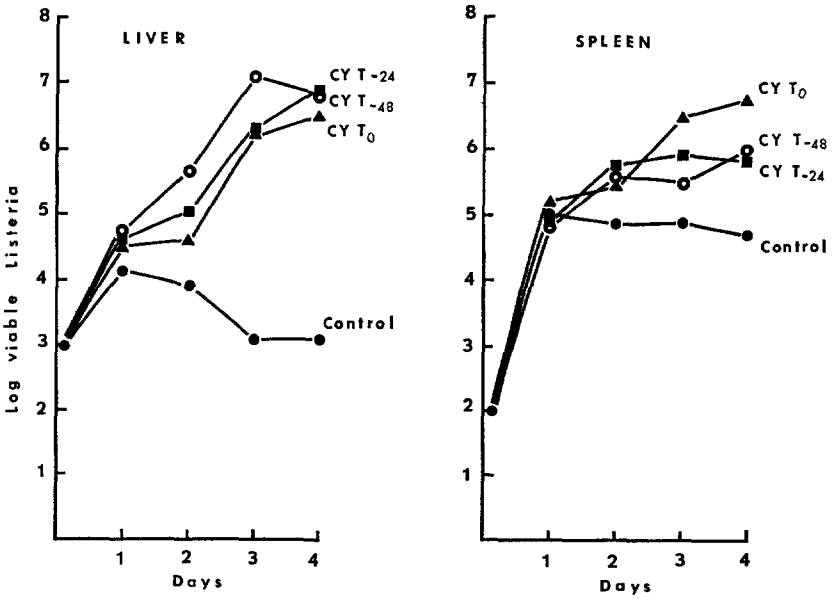

FIG. 5. Growth curves of *L. monocytogenes* in control mice and in mice given a single dose of cyclophosphamide at 48 (T_{-48}), 24 (T_{-24}), and 0 hr (T_0) prior to infection.

Time-Response Relationship.—It has been shown that a single dose of drug administered *at the time of infection* effectively suppressed the immune response to a *Listeria* infection. It was of interest to study next the immunosuppressive effects produced with a single dose administered at varying times before and after the initiation of infection.

The results of treatment with cyclophosphamide administered before or after the initiation of infection are contained in Figs. 5 and 6. They show that the

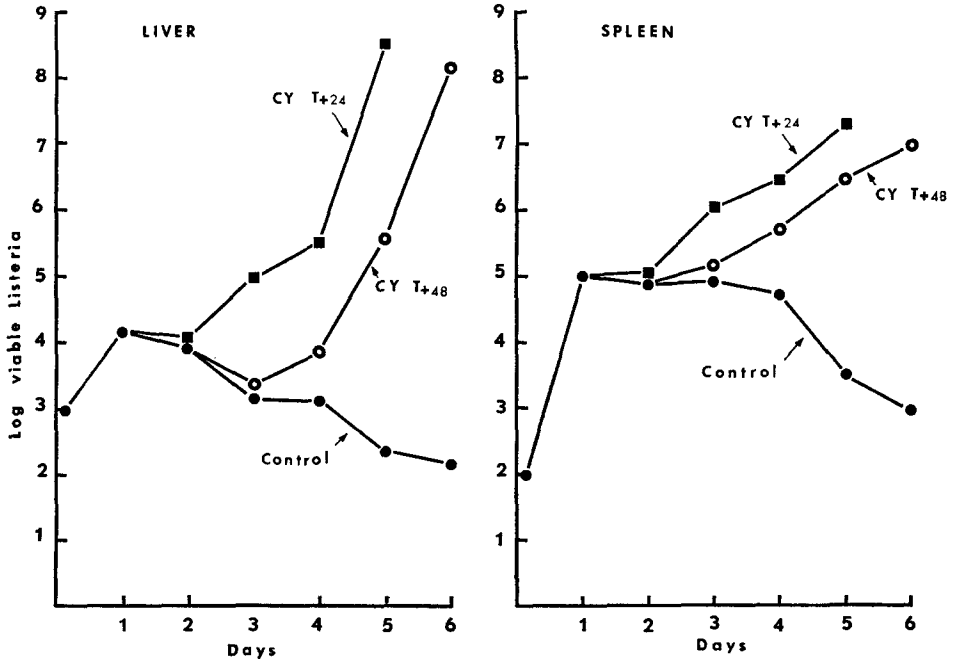

FIG. 6. Growth curves of *L. monocytogenes* in control mice and in mice given a single dose of cyclophosphamide at 24 (T_{+24}) and 48 (T_{+48}) hr after infection.

drug produced conspicuous impairment of the immune response when given as early as 48 hr before infection (Fig. 5). It was also capable of interfering with an ongoing response, as indicated by the curves obtained in animals treated 24 or 48 hr after infection (Fig. 6).

The data presented in Figs 5 and 6 can be used to express drug effects in terms of an *immunosuppressive index*; i.e., the difference between the log viable counts in drug-treated and control groups at any time. Since drug-treated animals do not always survive beyond 3 days of drug-host-parasite interaction, the bacterial counts at 72 hr were chosen for expressing the immunosuppressive index in the following studies with cyclophosphamide, vinblastine, and metho-

trexate. The experimental plan was similar to that used in the previous experiment.

Using the immunosuppressive index as defined above, the degree of immunosuppression obtained with a single injection of cyclophosphamide, vinblastine, and methotrexate administered at periods ranging from 11 days before to 7 days

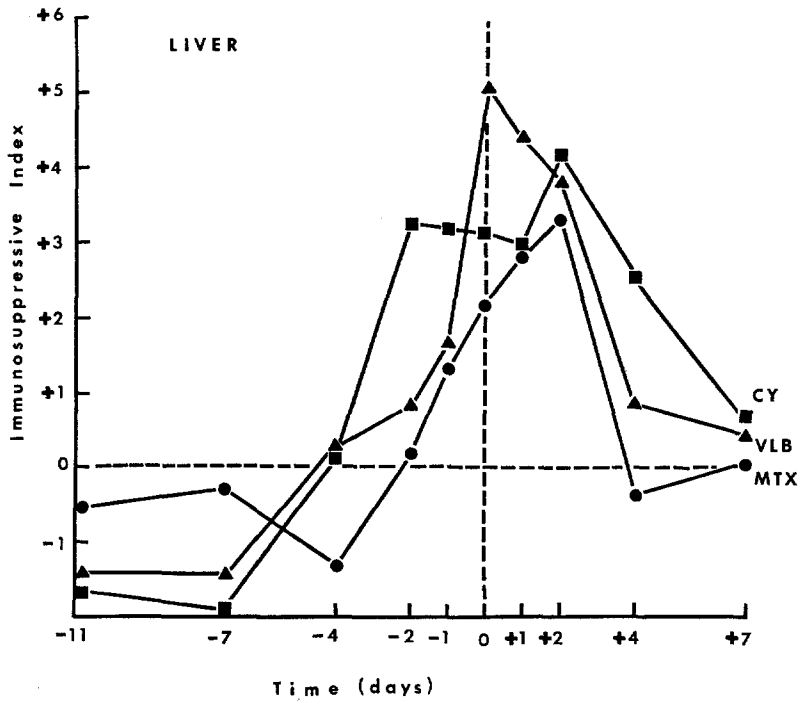

FIG. 7. Plots showing the differences in the immunosuppressive indices obtained in the liver when single doses of cyclophosphamide (CY), vinblastine (VLB), or methotrexate (MTX) were administered at varying times before (−11 to −1 days) or during (0 to +7 days) a *Listeria* infection.

after the initiation of infection was measured. The results are presented in Figs. 7 and 8. Considering first the effects observed in the liver, it is apparent that cyclophosphamide was immunosuppressive over a broad interval of time, from −2 to +4 days, its influence being virtually maximal during this period. Vinblastine, on the other hand, produced its greatest effect when given at the time of infection. It had only a slight influence when given prior to infection but was very active when given during the first 48 hr after infection. In the case of methotrexate, maximum activity was achieved in animals treated 48 hr after.

An interesting feature of the curves shown in Fig. 7 was the negative immuno-

suppressive index observed in animals treated with cyclophosphamide and vinblastine 7–11 days before infection, and a similar effect with methotrexate given 4 days before infection. This immunity-enhancing influence of immunosuppressive drugs when given prior to an immunological stimulus has been observed in other systems and will be discussed in greater detail later.

The immunosuppressive indices in the spleen (Fig. 8) were broadly similar but showed a tendency for the curves to shift to the right.

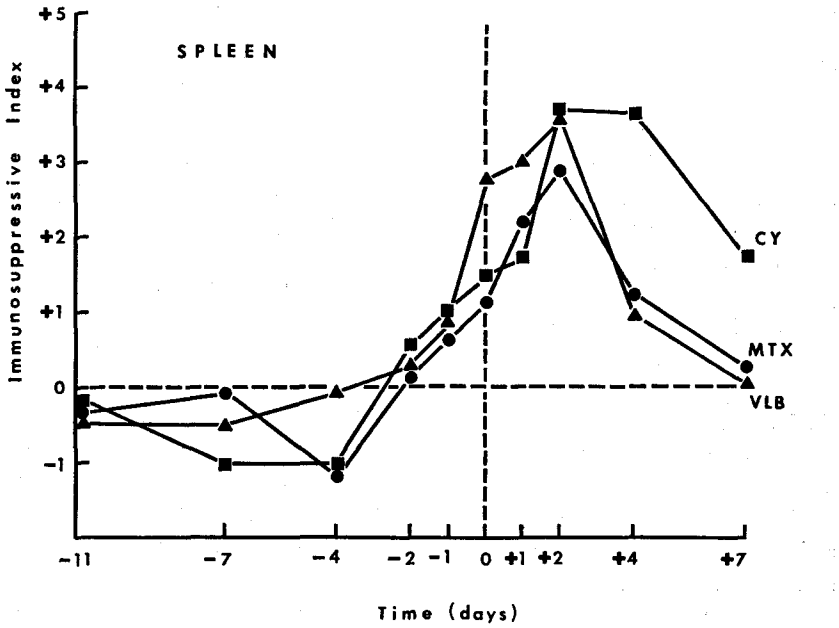

FIG. 8. Plots showing the differences in the immunosuppressive indices obtained in the spleen when single doses of cyclophosphamide (CY), vinblastine (VLB), or methotrexate (MTX) were administered at varying times before (−11 to −1 days) or during (0 to +7 days) a *Listeria* infection.

DISCUSSION

Many cytotoxic agents owe their immunosuppressive activity to their capacity to attack rapidly proliferating cells. Their action on humoral antibody production (1–4) or cell-mediated forms of immunity such as delayed-type hypersensitivity (5) and transplantation immunity (8, 9), have provided valuable information concerning the cellular events associated with these types of immune response. There have, however, been few studies concerned with their effect upon acquired resistance to infectious diseases, even though infection represents the major hazard associated with the use of such agents.

Our investigations have shown that administration of a single dose of any one of four broadly representative cytotoxic agents has profound effects on the course of *Listeria* infection in mice. It is of interest to consider what known effects of these cytotoxic agents could be held responsible for the results obtained. Since infection with intracellular parasites results in a cell-mediated form of immunity (10, 11), it is appropriate to discuss them in relation to reported findings of their action on delayed-type hypersensitivity and transplantation immunity.

The ultimate expression of resistance against intracellular bacterial parasites rests with the macrophage and not with the lymphoid cells which mediate the mechanism of immunity (10, 11). It is therefore possible that some of the effects observed might be due to interference with the activities of macrophages. That this is not a major factor is evidenced by findings, to be reported later,[2] which show that animals immunized with a heterologous organism (BCG) retain their resistance to challenge with *L. monocytogenes* after treatment with cyclophosphamide, vinblastine, or methotrexate. Since this nonspecific resistance is known to be due to activated macrophages, it is reasonable to conclude that none of these drugs has any major influence on the functional activity of macrophages. Moreover, 6-mercaptopurine, a purine analogue similar to azathioprine, does not influence the rate of clearance of heterologous erythrocytes from blood (12), nor does nitrogen mustard (believed by some to be the active form of cyclophosphamide) affect the particle clearing function of the reticuloendothelial system (13). These observations again indicate a lack of direct toxicity for the macrophage. It would appear therefore that most of the effects observed in the present study can be attributed to the influence of the drugs on the lymphoid cells which become engaged in the immune response leading to acquired resistance. More persuasive evidence on this point is presented in an accompanying paper (28).

There are at least three ways of interfering with an immune response: (*a*) depletion of immunocompetent cells by direct cytolysis; (*b*) impairment of cell metabolism; and (*c*) selective destruction, with mitotic poisons, of those cells which divide in the course of the immune response. The results obtained with the four drugs will be discussed in relation to these three possible modes of action.

The exact mechanism of induction of cellular immunity in listeriosis is as yet ill-defined. Present evidence indicates that it may be similar to the induction of delayed-type hypersensitivity (10, 11). Turk (5) has proposed that the steps leading to this type of hypersensitivity involve the sequence: small lymphocyte → immunoblast → immunologically committed small lymphocytes. That the immune mechanism in listeriosis is mediated through the lymphoid series of

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

cells and not by serum antibody, is indicated by recent studies from this laboratory which show that lymphoid cells from *Listeria*-immune mice are capable of conferring absolute immunity to *Listeria* challenge when transferred to normal recipients (10); and that specific antilymphocyte globulin abolishes the protection conferred in this way (11). The occurrence of pyroninophilic cells (similar to the pyroninophilic "immunoblasts" of Turk) in the spleens of mice during the early stages of infection[3] indicates that the evolution of the immune lymphoid cells in listeriosis could well involve the sequential stages: precursor cell → immunoblast → immunologically committed small lymphocytes. It is clear that immunosuppressive drugs could act on any of these three cell populations.

Cyclophosphamide, a member of the mustard series, belongs to a class of drugs designated as polyfunctional alkylating agents which have been shown to exert their toxic action on cells by forming crosslinks between double-stranded DNA, interfering with transcription and replication (14). Studies with ascites tumor cells have shown that the drug acts on the premitotic resting phase (G_2), as well as on the DNA-synthetic phase (S) of the cell cycle (15); it is toxic to precursor cells of both the erythroid and the lymphoid series (16). Nitrogen mustard (and presumably cyclophosphamide) is directly lethal for lymphocytes (17). It would seem therefore that cyclophosphamide has the potential to damage lymphoid cells at all stages of the immune response. Turk's investigations on delayed-type hypersensitivity suggest that it acts in three ways: it prevents the differentiation of small lymphocytes to immunoblasts; has a direct toxic action on immunoblasts; and is antimitotic. Our observations are consistent with this view since cyclophosphamide was found to have a profound effect on the host's resistance when given at any time from 2 days before to 4 days after infection, suggesting that it can affect the precursor cell, the intermediate cell, and possibly the immunologically committed small lymphocyte.

Vinblastine sulphate is known to act on dividing cells (18); it prevents the formation of the mitotic spindle, causing arrest in metaphase. Its effects are believed to be irreversible so that arrested cells ultimately die. The immunosuppressive effects obtained with vinblastine in the present study are therefore a reflection of the mitotic activity occurring during a normal immune response and thus throw some light on the cellular events which occur during the initial stages of the host's immune response to *L. monocytogenes*.

The slope of the normal growth curve from 12 to 24 hr (Fig. 1) indicates that the host's immune response is already manifest in the spleen at 24 hr. Radioautographic studies on spleens of *Listeria*-infected mice show marked mitotic activity at this time.[3] A relationship between this mitotic response and the de-

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[3] North, R. J. 1969. Cellular kinetics associated with the development of acquired cellular resistance. Submitted for publication.

velopment of immunity is indicated by the immunosuppressive effect obtained with a single dose of vinblastine administered *at the time of infection.* In fact, vinblastine was most effective when given at this time, suggesting that the presence of vinblastine for 15–18 hr (the duration of effective serum levels of vinblastine with the dosage employed) results in the deletion of *all* cells which have become mitotically engaged as a result of the immune response. This would interfere drastically with the natural transition from the precursor cell to immunologically committed small lymphocyte, and hold the host's acquired defenses in abeyance.

Azathioprine and methotrexate are antimetabolites which specifically interfere with the processes leading to the synthesis of DNA. Azathioprine prevents the conversion of inosinic acid to adenylic acid, an essential step in the synthesis of nucleic acids; it may, in addition, act through binding of sulfhydryl groups (1). Since a single dose of azathioprine, given at the time of infection, effectively suppresses the immune response (Fig. 3), its effects would seem to be irreversible in contrast to those of methotrexate (Fig. 2).

The most important biochemical lesion caused by methotrexate is a result of its profound influence on folic reductase. This enzyme reduces folic acid to dihydrofolic acid and tetrahydrofolic acid, thereby interfering with the formation of thymidine (19). The drug is believed to be bound irreversibly to the enzyme so that methotrexate treatment results in thymidine deficiency and impairment of DNA synthesis.

The present studies showed two features which distinguish the effect of methotrexate from those produced by other drugs. The time-response relationship showed that the immunosuppressive activity of methotrexate was highest when the drug was administered at 48 hr after infection, indicating that the drug probably acts most effectively at a later stage of the immune response. Turk (5) suggested from morphologic studies that methotrexate acts principally on the transformation of immunoblasts to immunologically committed small lymphocytes. North's studies[3] indicate that the maximum mitotic activity (predominantly involving immunoblasts) is found in the spleens at 48 hr after *Listeria* infection. If Turk's conclusions are applicable to the present model, it would be at this time that methotrexate would exert its greatest effect in suppressing the immune response to *Listeria*, a premise which is borne out by the findings; it is not clear, however, whether the immunosuppressive effect is due to interference with further cell division, cell differentiation, or both.

Another feature of interest was the reversible nature of immunosuppression with methotrexate. Since many tissues have reserves of tetrahydrofolic acid, the end-product of folic reductase activity, it is possible that after a single dose of methotrexate the reserves of tetrahydrofolate may be adequate to tide over the toxic effect of methotrexate toxicity. Under these conditions it is possible that there may be impairment of cell-function without death, and recovery may

ensue. It is also possible that the reversible nature of the immunosuppressive activity may be due to the persistence of some lymphoid cells with greatly elevated levels of folic reductase, a situation which has been encountered in some tumor cells which survive chemotherapy with methotrexate (20–22). The total immunosuppression achieved with two doses of methotrexate, while in favor of the former, suggests that the latter explanation is unlikely. The results thus indicate that cells can escape from the lethal action of this drug unless its levels are sustained.

The transient nature of methotrexate activity is also seen with other immune systems. Friedman and Buckler (23) observed that while methotrexate suppressed the induction of delayed-type hypersensitivity to tuberculin in guinea pigs, lymph node cells from these animals were capable of transferring sensitivity to normal recipients 2 wk later. The authors interpreted this finding to mean that methotrexate prevents the multiplication of sensitized cells but does not interfere with the induction of the immune response. The results of the present study give strong support to this view. Fig. 2 shows that bacterial populations in animals treated with methotrexate had reached levels higher than 10 million without any overt evidence of an immune response on the part of the host. Since this number of organisms is invariably lethal to mice (LD_{50} 5×10^3), yet the methotrexate-treated animals effectively controlled them from the 2nd day onwards, it seems that the immune response had proceeded in the presence of methotrexate to the extent that cells had been primed during the early stages; on being released from the effect of methotrexate, the primed cells must have undergone a sudden burst of activity, resulting in an effective immune response.

In the present study, pretreatment with three immunosuppressive drugs 4–7 days before infection actually resulted in enhancement of immunity. Similar effects on antibody production have been produced with cyclophosphamide (24), 6-mercaptopurine (6-MP) (25), and cytosine arabinoside (26). Chanmougan and Schwartz (25) observed that rabbits which had been treated with 6-MP and rested for 1 wk before challenged with small doses of antigen, produced levels of circulating antibody considerably higher than were achieved by untreated animals, an effect attributed to hyperplasia of the lymphoid system in the pretreated rabbits (27). Incidental observations in the present study also indicated that mice which have been treated with cyclophosphamide, vinblastine, or methotrexate show marked shrinkage of the spleen followed by gross enlargement and hyperplasia which was manifest 4 days after treatment with methotrexate and by the 7th day in animals treated with cyclophosphamide or vinblastine. Since the phase of hyperplasia coincided with the immunity-enhancing effect of the drugs, lymphoid hyperplasia may indeed explain this paradoxical effect of drugs which are normally immunosuppressive in their action. If these observations have any relevance to man, inadequate medication

during immunosuppressive therapy may actually enhance immunologic reactivity with possible adverse consequences. There is therefore a need for a precise knowledge of the time-dose relationship of each drug for ensuring optimal immunosuppressive effects.

The drugs used in the present study are among the most widely used in the chemotherapy of human cancer, and all but vinblastine are also used as immunosuppressive agents for the prevention of graft rejection and the control of autoimmune diseases. Since they were all found to interfere conspicuously with the development of resistance to a *Listeria* infection, it is not surprising that infection represents a major hazard in their clinical use. But the study has also shown that an experimental infection can be a very useful model with which to assess the immunosuppressive activity of drugs and the time-course of their action.

Analogies exist between the rapid proliferation of cancer cells and that associated with the inductive phases of an immune response. It is therefore logical to assume that drugs which will inhibit cancer cells may have a similar influence on the host response to infection, as was found in the case of the four anticancer agents used in this study. The method may perhaps be employed with advantage in the screening of anticancer agents, since the techniques are simple, objective, and quantitative.

SUMMARY

Four drugs, representing four different categories of cytotoxic agents, were studied for their effect on the immune response to *Listeria* infection in mice. The development of the host's immune response is revealed by a progressive change in the slope of the bacterial growth curve in spleen and liver. It has its onset at 24 hr in untreated mice, but in the presence of effective immunosuppression the organism multiples uninterruptedly until the animal dies from overwhelming infection. When administered as single injections at the time of infection, cyclophosphamide, vinblastine, and azathioprine all produced an effective immunosuppression, characterized by continuous bacterial multiplication.

Methotrexate was also immunosuppressive, but unlike the others its effects were reversible. They could be sustained, however, by further treatment.

Studies of the time-response relationship indicated that cyclophosphamide was highly active over a broad time-span ranging from 2 days before infection to 4 days after infection. Vinblastine on the other hand was maximally active when given on the day of infection, while methotrexate had its greatest effect when given 48 hr after infection. These differences indicate that these three drugs act on different cell populations involved in the host's immune response. The effects observed have been discussed in relation to what is known of the modes of action of the drugs tested.

An observation of interest was the phenomenon of enhanced immunity in animals treated with cyclophosphamide or vinblastine 7–11 days before, and with methotrexate 4 days before infection; reactive hyperplasia of lymphoid tissue following withdrawal of drug was again advanced as an explanation for the occurrence of this paradoxical effect.

The experimental model employed is simple, requiring only routine bacteriological facilities and minimal equipment. It seems to offer a useful means of assessing the immunosuppressive activity of drugs and of determining the time-course of their action; it could also be of value in the screening of anticancer agents.

The technical assistance of Mr. Jonathan Deissler is gratefully acknowledged.

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