EXACERBATION OF MURINE LISTERIOSIS BY A MONOCLONAL ANTIBODY SPECIFIC FOR THE TYPE 3 COMPLEMENT RECEPTOR OF MYELOMONOCYTIC CELLS

Absence of Monocytes at Infective Foci Allows *Listeria* to Multiply in Nonphagocytic Cells

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Protective immunity in mice to infection with the Gram-positive bacterium, *Listeria monocytogenes*, is mediated by *Listeria*-sensitized T cells, but expressed by macrophages (1). It is known (2, 3) that protective T cells are generated progressively between days 2 and 6 of a sublethal immunizing infection, and are lost progressively after this time, as active immunity is replaced by immunologic memory.

However, inactivation of *Listeria* begins on day 2 of infection, well before active immunity peaks, and it is associated with the accumulation of monocytes at foci of infection in the liver and spleen (4). It is generally assumed that blood monocytes are responsible for inactivating *Listeria* at foci of infection, and that inactivation is efficient only after monocytes are activated by sensitized T cells. It might be expected, therefore, that any agent that prevents monocytes from accumulating at sites of bacterial implantation would result in failure of the host to control bacterial multiplication.

An agent with likely capacity to interfere with the focusing of blood monocytes at infective foci in the liver and spleen is a recently described (5) mAb specific for the type 3 complement receptor (CR3)[1] of murine myelomonocytic cells. This rat mAb, designated 5C6, is specific for an epitope of the CR3 molecule that is different from the epitopes seen by other anti-CR3 mAbs (5). It profoundly inhibits the capacity of neutrophils and monocytes to emigrate from blood into peritoneal inflammatory exudates.

The purpose of this paper is to show that intravenous injection of 5C6 mAb completely prevents mice from protecting themselves against a sublethal inoculum of *Listeria*. It will demonstrate that the infection-promoting action of 5C6 is based on its capacity to interfere with the accumulation of monocytes at infective foci in the liver and spleen, thereby allowing the organism to multiply unrestrictedly in nonphagocytic cells.

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[1] *Abbreviation used in this paper:* CR3, complement receptor type 3.

Materials and Methods

Mice. AB6F$_1$ (AxC57BL/6) male mice were used when they were 10–12 weeks old. They were obtained from the Trudeau Institute Animal Breeding Facility, and were known to be free of common viral pathogens, as evidence by the result of routine screening by Charles Rivers Professional Services, Wilmington, MA.

Bacteria. A log-phase culture of *Listeria monocytogenes*, strain EGD, serotype 1/2a, was grown in Trypticase-soy broth, aliquotted in 1 ml volumes and stored at −70°C. The LD_{50} dose was 5×10^4 intravenously in AB6 mice. For each experiment a vial was thawed and diluted appropriately in PBS for intravenous inoculation in a volume of 0.2 ml, or for intradermal injection in a volume of 0.05 ml. The immunizing dose was 2×10^3. The organism was enumerated in the liver, spleen, and draining lymph node by plating 10-fold serial dilutions of homogenates of these organs on Trypticase-soy agar, and counting bacterial colonies after incubation for 24 h at 37°C. *Listeria* was enumerated at the site of intradermal inoculation in the belly by removing a 1-cm square piece of the abdominal wall, including the inoculation site. The tissue was cut into small pieces, homogenized in a blender at 10°C, and plated as described above. Clearance of an intravenous inoculum of *Listeria* from blood was followed by taking tail blood (0.02 ml) in heparinized capillary tubes at the times indicated, diluting the blood appropriately in PBS, subjecting the diluted blood to ultrasound to release intracellular bacteria, and plating 10-fold serial dilutions of the sonicate on nutrient agar as described above.

Monoclonal Antibodies. The 5C6 mAb specific for an epitope of the CR3 receptor of mouse myelomonocytic cells was generated by a rat B cell–rat myeloma hybridoma, as described previously (5). The mAb was purified by sodium sulphate precipitation, anion exchange, and exclusion chromatography, and the purity of the resulting IgG2b antibody assessed as described previously (5). The antibody was injected intravenously in a dose of 0.5 mg. The isotype-matched control rat mAb·IC5.5H10.A11 reacts with a surface and intracellular antigen restricted to murine myelomonocytic cells and immunoprecipitates a glycoprotein with a molecular mass of 200 kD nonreduced and 97 kD when reduced (Rabinowitz, S., and H. Rosen, unpublished observations). Sodium sulphate precipitate of ascites dialyzed into PBS and containing 0.5 mg of monoclonal IgG was injected intravenously in each case.

Histology. Mice were killed by cervical dislocation and perfused via the left ventricle with 10% buffered formalin solution. Their livers and spleens were then removed, cut into small pieces, and fixed for 24 h in the same buffered formalin solution. The tissue was then dehydrated in ethanol and embedded in glycol methacrylate (JB-4 embedding kit; Polysciences, Inc., Warrington, PA). Sections 1–2 μm in thickness were cut with glass knives and stained with toluidine blue. Microscopy was performed with a Nikon Microphot-Fx microscope.

Results

Intravenous Injection of 5C6 Prevents Mice from Controlling Bacterial Multiplication in their Livers and Spleens. The first experiment involved giving 5C6 intravenously to mice 24 h after inoculating them with a sublethal number (2×10^3) of *Listeria* via the same route. The rationale for giving 5C6 at 24 h into infection was to allow time for the organism to establish foci of infection in the liver and spleen. It is known (4) that at 24 h, sites of infection are populated predominantly by neutrophils.

It can be seen in Fig. 1 that a single 0.5-mg dose of 5C6 completely eliminated the capacity of mice to control bacterial multiplication in their livers and spleens. Whereas *Listeria* was progressively eliminated after day 3 of infection in control mice, it continued to multiply log linearly in the livers and spleens of 5C6-treated mice. The infection-promoting effect of 5C6 was obvious by 24 h after injection. All treated mice died by day 4 of infection, whereas all control mice survived. Treatment with IC5 mAb was also infection enhancing, but to a much smaller degree. Of the five mice kept to determine survival time, three were still alive on day 10 when the ex-

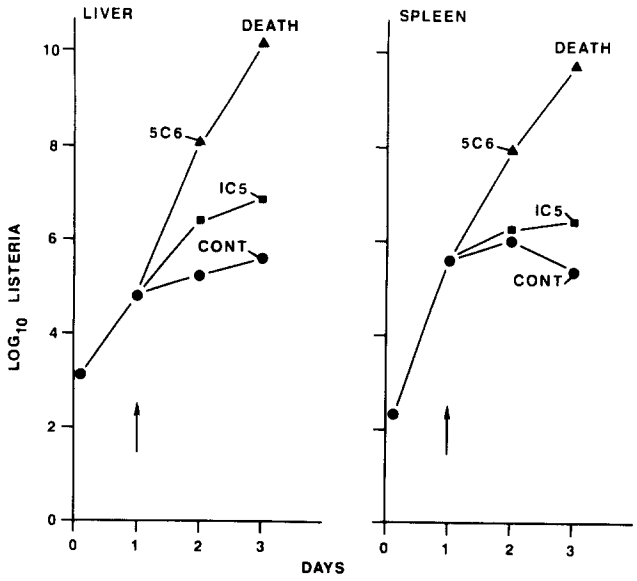

FIGURE 1. Intravenous injection of 0.5 mg of 5C6 mAb on day 1 (*arrow*) of a sublethal *Listeria* infection (5×10^3 intravenously) prevented mice from controlling *Listeria* multiplication in their livers (*left*) and spleens (*right*). All 5C6-treated mice died by day 4 of infection. Treatment of mice with the same quantity of IC5 mAb, however, caused much less enhancement of infection and three of five of the remaining mice survived. Means of five mice per group.

periment was ended. This much greater infection-enhancing effect of 5C6 will be obvious later.

5C6 Treatment Prevents the Accumulation of Monocytes and Neutrophils at Infective Foci and Allows Listeria to Multiply in Nonphagocytic Cells. It is known (4) that sites of *Listeria* infection in the liver and spleen are populated by neutrophils during the first 24 h of infection, and increasingly by monocytes after this time. Fig. 2 *a* shows the appearance in section of an infective focus in the liver of a control mouse on day 3 of a *Listeria* immunizing infection. It can be seen that the infective focus is populated predominantly by mononuclear cells, none of which can be seen to contain *Listeria*, because of the small total number of organisms present in the liver. Indeed, many sections had to be scanned, even to find infective foci. In contrast, infective foci were very numerous in sections of 3-d infected livers of 5C6-treated mice, indicating that *Listeria* had disseminated from the original sites of implantation to form additional foci of infection. More striking was the appearance (Fig. 2 *b*) of individual foci of infection in the livers of 5C6-treated mice. They were not populated by monocytes, as in control mice, but consisted of groups of infected hepatocytes in which *Listeria* had been multiplying unrestrictedly. By day 4 of infection foci of infection were much more numerous and larger, and were harboring enormous numbers of *Listeria*. It was obvious that infection was spreading contiguously from cell to cell, as well as via sinusoids to distant hepatocytes.

In the spleen, *Listeria* normally forms infective foci in the marginal zones of the white pulp. In control mice these sites of infection were predominantly populated by monocytes by day 3 of infection and contained very few visible *Listeria*. In 5C6-treated mice, in contrast, the white pulp was almost totally destroyed by day 3 of infection and was replete with extracellular *Listeria*. This will be the subject of a forthcoming paper (Rosen, H., S. Gordon, and R. J. North, manuscript in preparation).

It was obvious from the histological study that treatment with 5C6 made mice

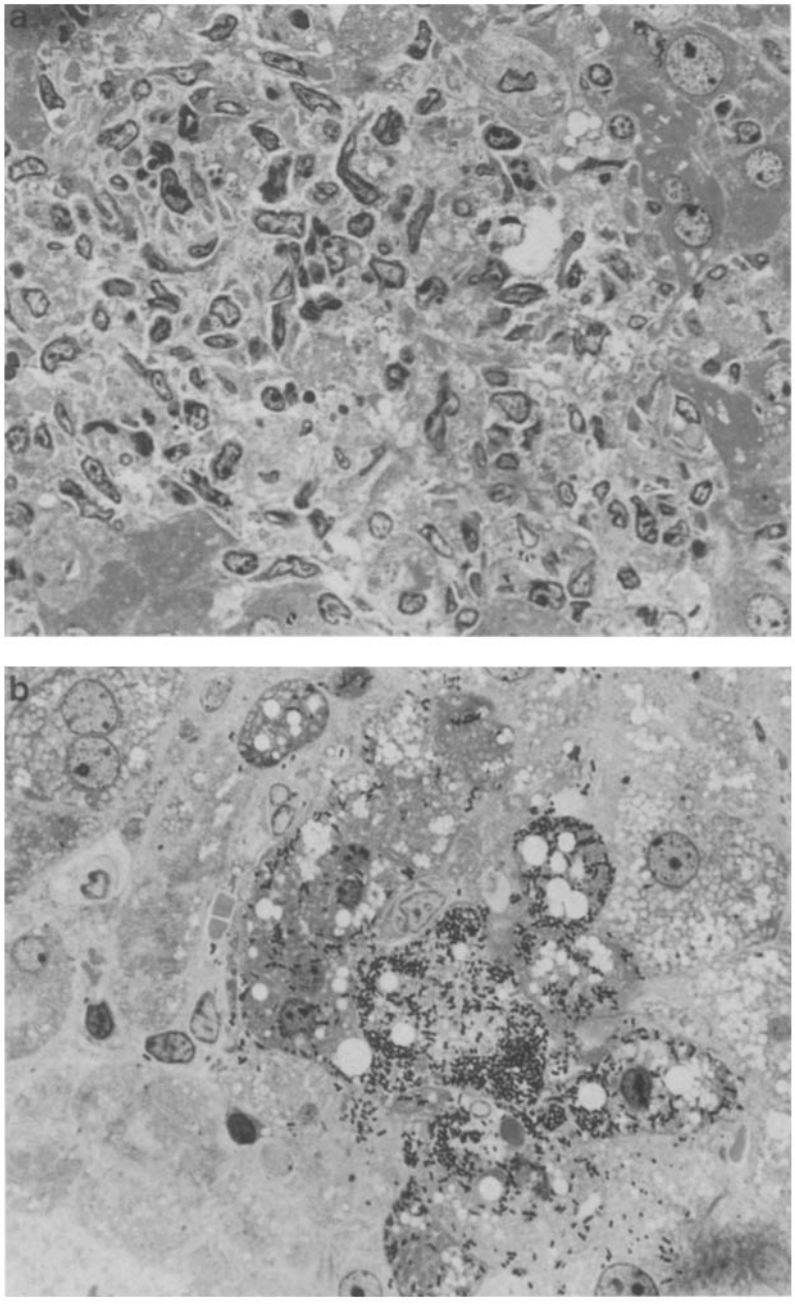

FIGURE 2. Plastic-embedded section of site of bacterial infection in the liver of a control mouse (*a*) and the liver of a 5C6-treated mouse (*b*) on day 3 of infection initiated with 5×10^3 *Listeria* intravenously. Whereas infective foci in control mice typically become populated by mononuclear cells, infective foci in 5C6-treated mice consist of groups of heavily infected hepatocytes with no obvious involvement of mononuclear phagocytes. ×900.

totally incapable of controlling bacterial multiplication and of confining bacteria to original sites of implantation. Consequently, the organism was free to disseminate from cell to cell, as well as via the blood to distant sites.

5C6 Has Only a Marginal Infection-enhancing Effect if Given after Day 3 of Infection. It was shown above that injecting 5C6 mAb on day 1 of a sublethal *Listeria* infection rapidly converted the infection to a lethal one, apparently because of a failure by the host to focus monocytes at sites of bacterial multiplication. It was important to determine next whether 5C6 is capable of promoting infection if given at a stage of infection when foci of infection are known to be already populated by monocytes, namely, between days 3 and 6.

Fig. 3 shows that, whereas injecting 5C6 on day 1 of infection resulted in log linear bacterial growth in the liver and spleen, injecting the same quantity of the antibody on days 4 or 6, when bacteria were already being inactivated in these organs, caused only a temporary promotion of infection. The former mice all died from infection, whereas the latter all survived.

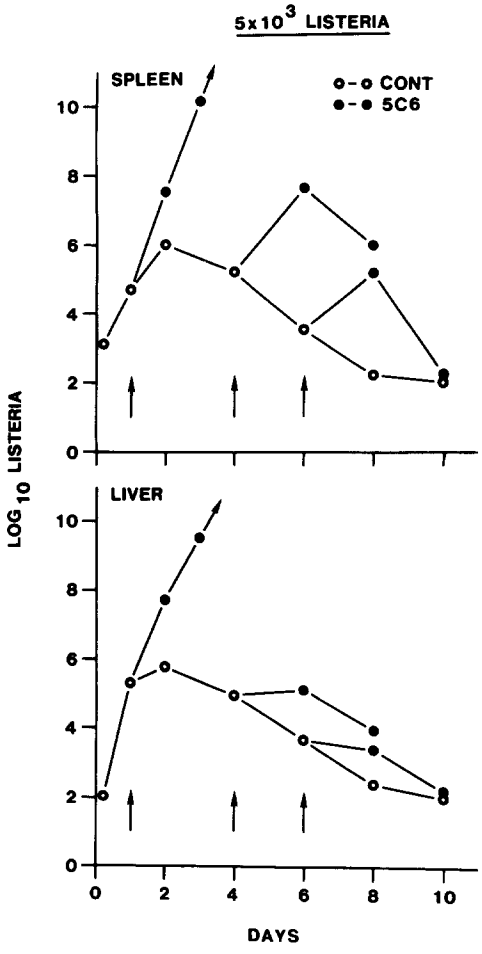

FIGURE 3. 5C6 treatment converted a sublethal *Listeria* infection to a lethal one if given on day 1 of infection, but not if given on days 4 or 6. Whereas 5C6 treatment on day 1 caused unrestricted log linear multiplication of *Listeria*, in the spleen and liver, 5C6 treatment on days 4 or 6 caused only a temporary increase in bacterial growth. Only those mice treated on day 1 died of infection. Means of five mice per group.

5C6 Does Not Inhibit the Capacity of Kupffer Cells to Remove Listeria from the Blood and Inactivate It. It is known (6) that 95% of an intravenous inoculum of *Listeria* is cleared by the liver, and that 50% or more of the cleared organisms are inactivated by Kupffer cells within 8–12 h. Because Kupffer cells express very low to undetectable levels of surface CR3 receptor (7), it was anticipated that treatment with 5C6 would not interfere with their ability to express this preimmunity antibacterial function in the liver.

Fig. 4 shows that intravenous injection of 5C6 mAb did not interfere with the ability of the liver to clear a *Listeria* inoculum given intravenously 1 h later. In control and treated mice an inoculum of 5×10^5 bacteria was essentially removed from the blood within 1 h, with >90% being cleared by 15 min. Fig. 5 shows that most of the inoculum was cleared by the liver, and that 90% of the cleared bacteria was inactivated in ~6–8 h. Treatment with 5C6 had no effect on the inactivation of *Listeria* during this time. However, 5C6 treatment promoted *Listeria* multiplication soon after, resulting in 10 times more bacteria in the liver at 24 h, and 3 logs more (result not shown) at 48 h. 5C6 had no effect on bacterial multiplication in the spleen during the first 24 h, but caused log linear growth in this organ after 24 h (results not shown).

5C6 Treatment Renders Mice 10^6 Times More Susceptible to an Intradermal Listeria Inoculum. It was evident from the histological study described above that treatment with 5C6 prevents mice from containing bacteria at original sites of bacterial implantation and of destroying bacteria therein. It was considered likely, therefore, that the infection promoting action of 5C6 would be even more strikingly demonstrated in mice inoculated with *Listeria* at a single site intradermally.

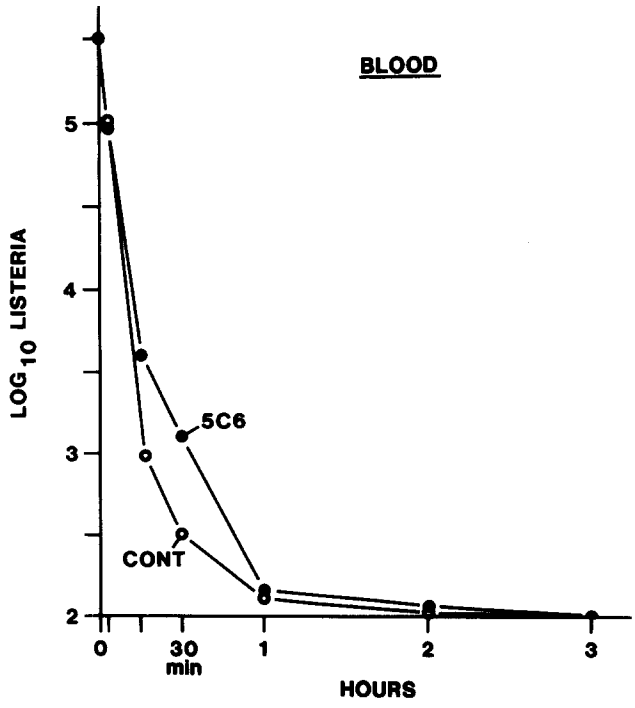

FIGURE 4. Clearance of *Listeria* from the blood of control mice and mice treated with 5C6 1 h before intravenous inoculation of 5×10^5 bacteria. Enumeration of bacteria in tail vein blood at the times indicated shows that there was no significant difference between control and 5C6-treated mice in the rate at which *Listeria* was removed from blood. Results with pooled blood from five mice per group.

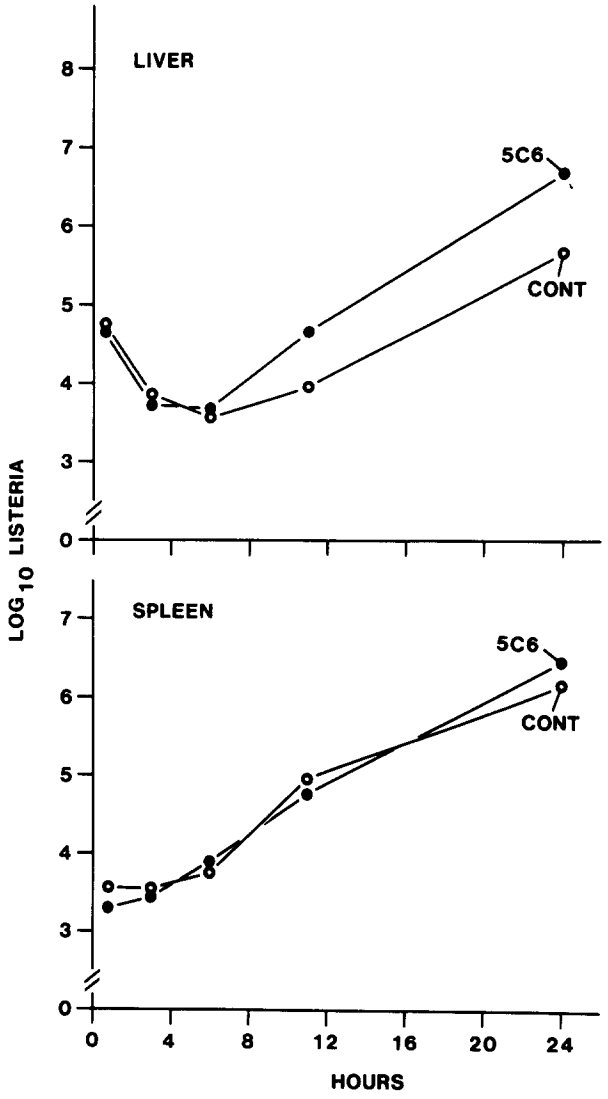

FIGURE 5. Treatment with 5C6 mAb 1 h before inoculating mice intravenously with 10^5 *Listeria* had no effect on the ability of liver macrophages to destroy a large proportion of the bacterial inoculum during the first 8 h of infection. The infection-promoting effect of 5C6 treatment was evident in the liver, but not in the spleen, at 24 h of infection.

It was found (Fig. 6) that normal mice are very resistant to *Listeria* inoculated intradermally, in that they survived an inoculum of at least 10^7 bacteria. In contrast, mice treated intravenously with 5C6 1 h earlier all succumbed to an inoculum of as few as 10 bacteria. Injecting IC5 intravenously 1 h earlier, however, had no effect on the ability of mice to resist intradermally inoculated *Listeria*.

The reason for this striking infection-promoting action of 5C6 is indicated by the results of an experiment that measured the growth of a 5×10^3 intradermal *Listeria* inoculum at the site of inoculation, and in the draining lymph node, liver, and spleen of control and 5C6-treated mice. It can be seen in Fig. 7 that 5C6 treatment enabled *Listeria* to disseminate more rapidly to the liver and spleen and to multiply in these organs without restriction. This interference with the ability of the host to contain

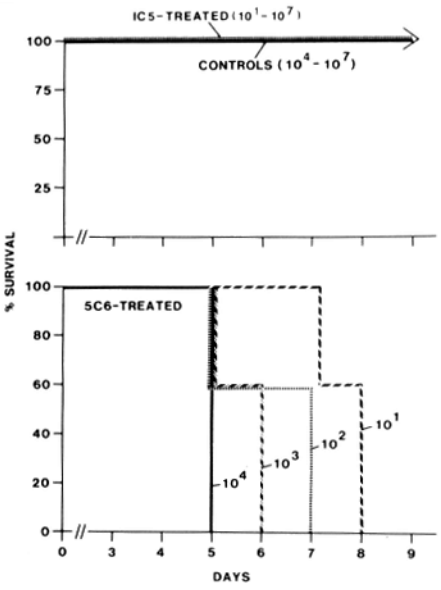

FIGURE 6. Evidence that treatment with 5C6, but not IC5, mAb greatly promoted infection in mice inoculated with *Listeria* intradermally. Whereas, not even as many as 10^7 *Listeria* given intradermally were lethal for control AB6 mice or IC5-treated mice (*top*), as few as 10 *Listeria* inoculated intradermally killed all mice treated with 5C6 mAb 1 h before infection.

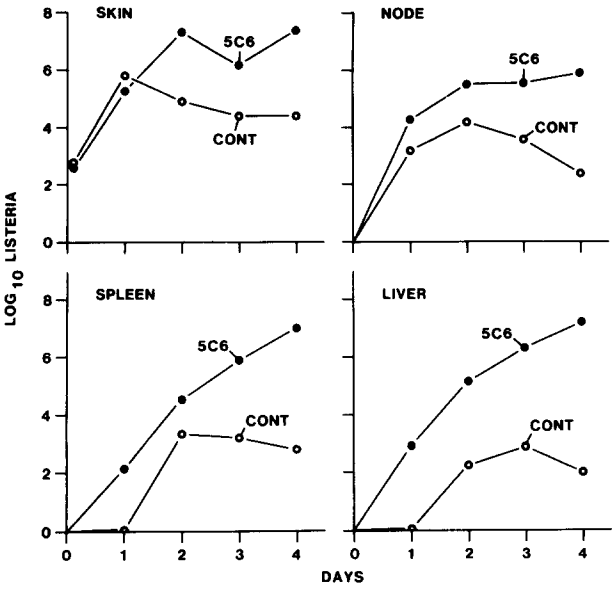

FIGURE 7. Consequence of 5C6 treatment on multiplication and dissemination of 5×10^3 *Listeria* inoculated intradermally. 5C6 treatment 1 h before infection caused increased multiplication of *Listeria* at the site of inoculation and in the draining lymph node (axillary). However, the most significant infection-enhancing effect of 5C6 treatment was seen in its ability to promote earlier dissemination of *Listeria* to the liver and spleen, and multiplication of the organism in these organs. Means of five mice per group.

the organism at the site of inoculation seemed more detrimental to the host than increased multiplication of the organism at the site of inoculation and in the draining lymph node.

Discussion

This study shows that intravenous injection of a mAb, designated 5C6, specific for an epitope of the CR3 receptor of murine myelomonocytic cells prevents mice

from defending themselves against a sublethal, intravenous inoculum of *Listeria monocytogenes*. Whereas control mice were able to progressively eliminate the organism from their livers and spleens after days 2–3 of infection, 5C6-treated mice were incapable of exercising any control over bacterial multiplication. Consequently, all treated mice succumbed to overwhelming infection within 5 d. This striking infection-promoting action of 5C6 was even more convincingly demonstrated in mice infected with *Listeria* intradermally. AB6 mice (and almost certainly other strains) are highly resistant to *Listeria* inoculated intradermally, in that they survive an inoculum of 10^7 at this site without any outward signs of illness. However, AB6 mice treated with 5C6 1 h before being inoculated intradermally all succumbed to as few as 10 organisms. It was apparent from the bacterial growth curves that 5C6 treatment exacerbated infection, not only by reducing the ability of the host to control bacterial multiplication at the site of inoculation, but by neutralizing its ability to retard the dissemination of bacteria from the site of inoculation to the liver and spleen. Consequently, *Listeria* reached the liver and spleen much sooner, and once implanted in these organs multiplied without restriction.

The infection-enhancing effect of a different anti-myelomonocytic cell mAb (IC5) was not nearly as impressive. While the control mAb caused some increased multiplication of intravenously inoculated *Listeria*, though less than that caused by 5C6, it had no obvious effect on the ability of mice to resist infection caused by intradermally inoculated *Listeria*. IC5 does not impair the recruitment of macrophages to thioglycollate broth in the peritoneal cavity, and the mild exacerbation of the intravenously inoculated *Listeria* may reflect some other impairment of the macrophage–organism interaction once the macrophage has already been recruited to the infected focus.

A histological examination of the livers of 5C6-treated mice infected intravenously with an otherwise sublethal inoculum of *Listeria* revealed that increased bacterial multiplication in this organ was associated with a failure of monocytes and other leukocytes to accumulate at sites of bacterial implantation. Therefore, instead of sites of infection being seen as macrophage-populated granulomas containing very few visible *Listeria*, foci of infection in 5C6-treated mice consisted of macrophage-free islets of infected hepatocytes in which *Listeria* had been multiplying extensively. These foci of infected hepatocytes became larger and more numerous with time, and contained enormous numbers of bacteria. It was obvious that the organism was spreading from one hepatocyte to the next and to distant hepatocytes via the sinusoids. By day 3 of infection a major proportion of liver hepatocytes were overwhelmingly infected with *Listeria*. It was apparent that 5C6 treatment had totally eliminated the host's capacity to control bacterial multiplication and dissemination.

There seems to be little doubt from this study that hepatocytes, rather than macrophages, are the main targets of *Listeria* in the liver, and that hepatocytes are devoid of a capacity to inactivate this pathogen intracellularly. It has been shown (8), in this connection, that *Listeria* is also capable of entering and rapidly multiplying in murine fibroblasts and other cells (9, 10) in vitro, and it is reasonable to suspect that these cells are also capable of supporting the growth of *Listeria* in vivo. Indeed, rather than being able to support the growth of *Listeria*, as is generally assumed, it is apparent that the cells in the mouse that predominantly kill *Listeria* are macrophages. Moreover, it has been demonstrated that normal, as well as activated mac-

rophages, are able to rapidly ingest and inactivate *Listeria* in vitro (11). The evidence that macrophages are responsible for destroying *Listeria* in vivo consists of the demonstration (4) that progressive inactivation of the organism in the liver and spleen does not begin until after day 2 of infection when macrophages progressively replace neutrophils at foci of infection. Therefore, the absence of macrophages at sites of bacterial multiplication in the liver and spleen of 5C6-treated mice means that the only host cell with a known capacity to destroy *Listeria* is missing from sites at which anti-*Listeria* resistance needs to be expressed. Thus, the infection-promoting action of 5C6 can be explained wholly on the basis of a failure of the host to focus macrophages at sites of infected hepatocytes at an early enough stage of infection. If *Listeria* is allowed to multiply for a number of hours in these cells without restriction, a sublethal number of *Listeria* is rapidly converted to a lethal number that the host is incapable of destroying, even if it eventually regains the capacity to direct macrophages to sites of infection.

If, on the other hand, foci of infection are already populated by macrophages before 5C6 is given, the situation is entirely different, in that 5C6 causes only a temporary increase in bacterial number. Presumably at this stage of infection, foci of infected hepatocytes are already surrounded by enough macrophages to contain the infection and destroy the organism at these sites. Whether 5C6 has the capacity to interfere with ingestion and destruction of *Listeria* by monocyte-derived macrophages at infective foci has yet to be determined. 5C6 does not have the capacity to interfere with the ability of liver Kupffer cells to clear an intravenous inoculum of *Listeria* from the blood, or to interfere with the ability of Kupffer cells to destroy, in 6–8 h, a large proportion of the *Listeria* load they ingest.

The infection promoting action of 5C6, then, would appear to be based on its ability to block a crucial defence mechanism that operates between 12 and 36 h of infection, namely, the accumulation of blood monocytes at foci of bacterial multiplication. The ability of 5C6 to interfere with the focusing of monocytes at foci of infection is in keeping with its ability to prevent the migration of myelomonocytic cells from blood into peritoneal inflammatory exudates and to sites of delayed hypersensitivity as described previously (5, 12).

It should be noted that exacerbation of infection by the 5C6 mAb is specific for acute bacterial pathogens such as *Listeria* and that 5C6 fails to potentiate infections of a number of different murine pathogens including West Nile virus, *Plasmodium Yoelii*, and *Candida albicans* (Rosen, H., and S. Gordon, unpublished observations). It is also possible that a variety of other mAbs directed to the macrophage surface might potentiate listeriosis, for example, by impairing phagocytic uptake or killing without inhibiting myelomonocytic recruitment to infective foci.

Summary

Treatment of mice with a rat mAb (5C6) specific for an epitope of the type 3 complement receptor of myelomonocytic cells severely interfered with the ability of the mice to resist infection with *Listeria monocytogenes*. Consequently, a sublethal infection was rapidly converted to a lethal one that resulted in death in 5 d. However, infection was only exacerbated if 5C6 was given earlier in infection, before mononuclear phagocytes populated sites of *Listeria* implantation in the liver and spleen. If given after day 3 of infection, 5C6 caused only a temporary increase in bacterial multipli-

cation. The infection-enhancing effect of 5C6 was associated with failure of mice to focus mononuclear phagocytes at sites of bacterial multiplication of *Listeria* in liver hepatocytes and extracellularly in the spleen. This resulted in unrestricted multiplication of *Listeria* in hepatocytes and extracellularly in the spleen. The results are in keeping with the ability of 5C6 to inhibit the accumulation of myelomonocytic cells in peritoneal inflammatory exudates, as revealed by a previous study (5).

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