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Host Defenses in Experimental Scrub Typhus: Effect of Chloramphenicol

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The effect of chloramphenicol treatment on the development of immunity to scrub typhus in mice was studied. Chemotherapy was administered either shortly before infection and for 14 days thereafter (group I), or from 7 to 21 days postinfection (group II). Although the full course of either regimen resulted in complete protection of the mice against subsequent challenge with the homologous strain of *Rickettsia tsutsugamushi*, initiation of chemotherapy at 7 days postinfection resulted in more rapid development of immunity against both the original infection and subsequent challenge. In both treatment groups, a 1- to 2-day hiatus was observed between immunity to challenge in the treated animal and the ability to transfer this immunity to syngeneic recipients with lymphocyte-enriched spleen cells. Similarly, complement-fixing antibodies were not detectable until shortly after the animals were able to resist challenge. These data supported the conclusion that the rickettsiostatic effect of chloramphenicol allows the infected animal time to mount an effective immune response and, further, that initiation of chemotherapy early in the infection may delay development of this response.

The efficacy of chloramphenicol as a chemoprophylactic and chemotherapeutic agent in the treatment of natural scrub typhus infections in humans and experimental infections of rodents is well established (2, 7, 12, 13, 15-17). In the experimental situation, appropriate treatment of infected mice not only resulted in their survival but also rendered such animals immune to subsequent lethal challenge by homologous or heterologous strains of *Rickettsia tsutsugamushi* (12). Experimental evidence from laboratory infections of mice (12) and circumstantial evidence from natural infections of humans (5, 8, 9, 11, 15) have suggested that reduction of the relapse rate after withdrawal of chloramphenicol therapy was related to the development of a protective immune response.

This laboratory has recently described the development of protective heterologous immunity in mice to infection with *R. tsutsugamushi*. We have shown that resistance in the early stages of primary scrub typhus infection was due principally to the development of cellular immunity, with the role of circulating antibody remaining undefined (10). Considering the effectiveness of chloramphenicol in treatment of scrub typhus infections, it was of interest to study the effects of this drug on the development of immunity in primary infections. In this report, we have correlated the length of chloramphenicol treatment and the time of initiation of treatment after infection with the subsequent development of cellular and humoral immune responses.

MATERIALS AND METHODS

Animals. Female BALB/c mice (Flow Laboratories, Dublin, Va.), 18 to 22 g, were used throughout the study.

Rickettsial strain. The Karp strain (egg passages 53 and 56) of *R. tsutsugamushi* was propagated, stored, and quantified by methods previously reported (1).

Chloramphenicol treatment of mice. Chloramphenicol (Parke, Davis & Co., Detroit, Mich.) solution was prepared in distilled water to a final concentration of 2.5 mg/ml and given to mice as their drinking water. Fresh solution was given to the animals twice weekly. Two basic regimens of chloramphenicol treatment were used—group I: drug was given to mice 2 days before Karp inoculation and maintained through day 14 postinoculation; group II: drug was given 7 through 21 days post-Karp inoculation.

Preparation of spleen cells. Mice to be used for cell transfer studies were killed by cervical dislocation. Spleens were removed aseptically and minced into

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small pieces. Fragments were pressed through a stainless-steel, 60-mesh screen into a plastic petri dish (60 by 15 mm; Falcon Plastics, Oxnard, Calif.), containing L-15 medium (Microbiological Assoc., Bethesda, Md.) supplemented with 2% heat-inactivated fetal bovine serum. Cell suspensions were washed twice with Earle balanced salt solution.

Separation of adherent and nonadherent cells. Freshly harvested and washed spleen cells in RPMI 1640 (Microbiological Assoc., Bethesda, Md.) containing 10% heat-inactivated fetal bovine serum were incubated in a plastic tissue culture flask (75 cm^2; Falcon Plastics, Oxnard, Calif.) for 1 h at 37°C in a humidified atmosphere containing 5% CO_2 in air. The leukocytes that did not adhere to the plastic surface after incubation were aspirated and, by morphological criteria, consisted of more than 95% lymphocytes. Suspensions were adjusted to contain 30×10^6 cells per 0.2 ml, and viability was monitored by trypan blue exclusion.

Preparation of cell homogenates. After spleen lymphocytes were adjusted to the appropriate concentration, cells were homogenized for three 1-min cycles in a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.).

Serological response. Mice treated in accordance with each chloramphenicol regimen were bled weekly from the retro-orbital venous plexus, and specimens from each bleeding were pooled according to regimen. Antibody was determined by the complement fixation test, using the CF52 method (6) adapted to microtiter apparatus. Strain-specific antigens were prepared by the method of Elisberg et al. (3).

Injection of animals. Mice received all injections of rickettsiae, cells, and homogenates by intraperitoneal injection in a standard volume of 0.2 ml.

RESULTS

Effect of chloramphenicol on mouse survival after lethal Karp challenge. When mice were maintained on antibiotic for the full duration of each regimen, complete survival was observed. Infected, untreated animals all died, usually within 10 to 13 days after challenge.

Effect of withdrawal of chloramphenicol treatment on mouse survival after lethal Karp challenge. The duration of antibiotic treatment necessary for protection in each regimen was investigated by withdrawing the drug on successive days after initiation of treatment. Chloramphenicol was first withdrawn on day 3 of treatment in group I and after day 1 of treatment in group II (Fig. 1).

Group I mice received chloramphenicol in their drinking water from 2 days before infection until 14 days after infection with 1,000 50% mouse lethal doses (MLD_{50}) of Karp and failed to survive if the drug was withdrawn before 11 days of postinfection therapy (Fig. 1). If the antibiotic was removed 11 to 13 days after infection, some mice did survive, and, by day 14, no mortality was observed. Although ultimate death of animals was not affected by the number of days of antibiotic treatment during the initial 10 days after infection, some effect of the drug was noted, as the length of survival before death increased proportionately with the duration of drug treatment during this period (Table 1). However, subtraction of the number of days of treatment from the total days of survival reveals that the mice invariably succumbed after approximately 13 days of uninhibited rickettsial proliferation. Animals surviving antibiotic withdrawal were challenged with 1,000 MLD_{50} of Karp 35 days after termination of treatment and found to be immune.

Animals in group II received chloramphenicol from 7 to 21 days after Karp infection. In this

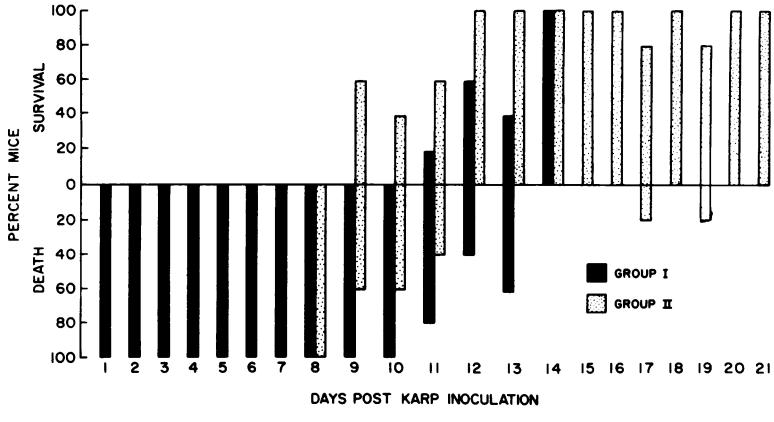

FIG. 1. *Effect of time of initiation of chloramphenicol treatment and duration of therapy on ability of mice to survive infection with the Karp strain of R. tsutsugamushi. Chloramphenicol therapy was initiated 2 days before infection in group I and 7 days after infection in group II. Each bar indicates the fate of a separate group of five mice that were withdrawn from drug therapy on the day indicated.*

case, some mice survived when antibiotic was withdrawn after only 2 days of treatment, and complete protection was first observed 12 days after infection, or 5 days after initiation of treatment. Despite the early appearance of survivors, occasional deaths were noted in this group when the drug was withdrawn at times subsequent to initial observation of complete protection (days 17 and 19 postinfection; Fig. 1). Survival time of dying mice was prolonged, and survivors were resistant to subsequent challenge as observed in group I (Table 1).

Effects of chloramphenicol on the infectious burden of spleen. Another means of assessing efficacy of a particular antibiotic regimen was by assay of the infectious rickettsial burden in spleen cells. To investigate this effect, 30×10^6 nonadherent spleen cells were obtained from each group of mice on selected days and briefly homogenized. The disrupted cells were transferred to recipient mice either undiluted (30×10^6 per 0.2 ml) or after serial dilution, which subsequently allowed calculation of the rickettsial MLD_{50} of the transferate. Inspection of Fig. 2 indicated a constantly increasing infectious burden in spleens of group I mice that reached a level of 6.0×10^4 MLD_{50} of Karp per 30×10^6 cells at 28 days postinfection. Quite the opposite effect was seen in group II mice, in which the initial antibiotic-free period of rickettsial growth was reflected in 6.3×10^4 MLD_{50} of rickettsiae recoverable at an early time, but very few were detectable at 28 days. It is clear that group II, which allows an early replication period for the rickettsiae, was more effective in producing survival in the shortest period of time (see above) and also in reducing the spleen's rickettsial burden.

TABLE 1. *Length of survival after withdrawal of chloramphenicol*

Drug treatment (days)	Survival for group:[a] I	II
1	14 (± 0.6)	13 (± 0.4)
2	15 (± 0.0)	14, 14; 60% survival
3	16 (± 0.4)	12, 12, 21; 40% survival
4	18 (± 0.5)	13, 15; 60% survival
5	17 (± 1.3)	100% survival
6	20 (± 0.4)	100% survival
7	21 (± 0.2)	100% survival
8	22 (± 1.0)	100% survival
9	23 (± 0.3)	100% survival
10	23 (± 0.6)	26; 80% survival
11	24, 24, 24, 26; 20% survival	100% survival
12	25, 29; 60% survival	26; 80% survival
13	26, 27, 27; 40% survival	100% survival
14	100% survival	100% survival

[a] When all animals in each group of five died, the mean day of death ± standard error of mean is indicated. When some animals survived, the day of death of each of the remaining animals is listed.

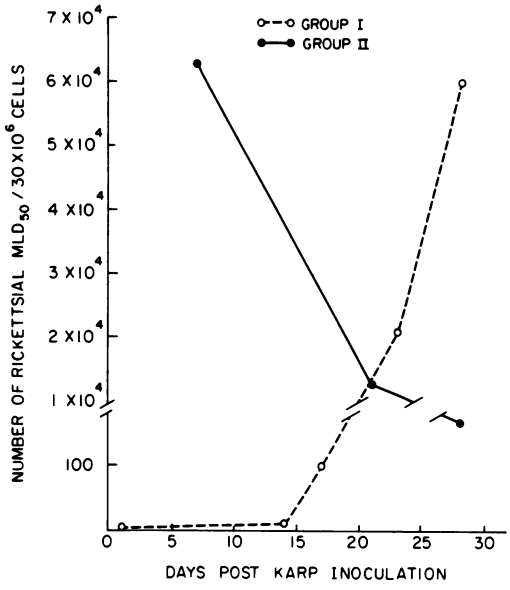

FIG. 2. *Effect of chloramphenicol regimen on the number of MLD_{50} of rickettsiae present in nonadherent spleen cells from mice infected with the Karp strain of R. tsutsugamushi. Mice in both groups were maintained on antibiotic for the full duration of each regimen, and all animals survived infection.*

Antibody production. Production of complement-fixing antibody by mice included in the 2 groups was different with respect to time of appearance in the serum and maximum titer achieved (Fig. 3). The mice in group I required 3 weeks to develop a detectable response, but evidenced a relatively high titer (1:160) by 5 weeks and maintained antibody levels in the range of 1:80 to 1:160 through 15 weeks. Mice in group II had a 1:20 titer 14 days after inoculation of rickettsiae and a constant increase in complement-fixing antibody level until a titer of 1:160 was achieved in week 4. Serum titer was reduced to 1:40 by week 5 and remained in the range of 1:40 to 1:80 for the remainder of the 15 weeks.

Resistance of drug-treated mice to rechallenge and ability to passively transfer protection with spleen cells. The capacity of surviving mice in each treatment group to withstand rechallenge with 1,000 MLD_{50} of Karp at time of drug withdrawal is shown in Table 2. In

addition, the ability of such survivors to passively confer protection by transfer of 30×10^6 splenic lymphocytes to recipient mice is also indicated. The group I mice were assayed 17 days after Karp infection (3 days after completion of antibiotic treatment) and subsequently at 2-day intervals. Three days after drug withdrawal, 80% of the potential donors withstood secondary Karp challenge, but their spleen lymphocytes were unable to transfer protection. By day 19, the immune protection observed in donors was transferable with spleen cells, and a substantial level of protection was observed in recipients for the remainder of the experiment, which was concluded 28 days postinfection. By contrast, group II mice withdrawn from chloramphenicol 10 days after infection, with only 3 days of drug treatment, evidenced complete protection against Karp, with development of cell-transferable resistance emerging shortly thereafter. Thus, use of the treatment regimen in which proliferation of rickettsiae initially was unaffected by antibiotic resulted in early development of immunity and rapid attainment of the ability to transfer protection with splenic lymphocytes.

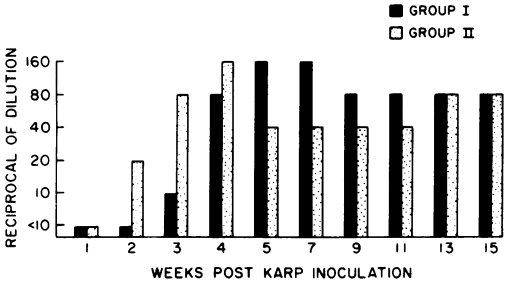

FIG. 3. *Effect of chloramphenicol regimen on the development of complement-fixing antibodies by mice infected with the Karp strain of R. tsutsugamushi.*

DISCUSSION

Clearly this study shows that the efficacy of chloramphenicol treatment for primary Karp infection of mice depended in great part on the time of initiation of treatment. The mice in group I were treated with chloramphenicol before infection, which inhibited replication of the rickettsiae until withdrawal of the drug. A substantial number of these mice succumbed to the initial infection if withdrawn from chemotherapy before 14 days of treatment, at which time they were immune to rechallenge with Karp. After completion of drug treatment, rickettsial proliferation commenced, as indicated by the increased infectious burden in the spleen, and shortly thereafter both detectable complement-fixing antibodies and cell-transferable immunity were observed. On the other hand, rickettsial proliferation was allowed to proceed for 7 days in group II mice before initiation of chemotherapy. The rickettsial infectious burden in the spleen was high when chemotherapy was initi-

TABLE 2. *Effect of chloramphenicol on the ability of Karp-infected mice to withstand rechallenge or to passively transfer protection with spleen cells*

Group	Days after Karp infection[a]	Days after completion/initiation of chloramphenicol treatment	Survivors (%)	
			Potential donors after challenge[a,b]	Spleen cell recipients challenged with Karp[b,c]
I	17	3 (C)[d]	80	0
	19	5 (C)	80	80
	21	7 (C)	100	100
	23	9 (C)	100	80
	25	11 (C)	100	100
	28	14 (C)	100	100
II	10	3 (I)	100	0
	11	4 (I)	100	80
	12	5 (I)	100	80
	16	9 (I)	100	100
	24	3 (C)	100	100
	28	7 (C)	100	100

[a] Infection and challenge doses consisted of 1,000 MLD_{50} of the Karp strain.

[b] Potential donor group for each time point consisted of 10 mice infected with Karp and protected with chloramphenicol. Five mice were challenged with Karp, and the remaining five were sacrificed to serve as spleen cell donors.

[c] These animals received 30×10^6 lymphocytes from the indicated donor group, followed by Karp challenge 8 h later.

[d] C, Days after completion of drug treatment; I, days after initiation of drug treatment.

ated, but decreased continuously through the 28-day observation period. These mice could be removed from therapy after a very short course with little recrudescence of lethal infection. This regimen also fostered more rapid production of serum antibody and cell-transferable protection, both of which became evident approximately 1 week before their appearance in group I mice.

The complement-fixing antibody titers ultimately achieved in infected mice were independent of the type of chloramphenicol regimen. After initial detection of antibody in each group of mice, the response kinetics were similar. However, detectable antibody appeared 1 week earlier in group II mice, with the result that titers in this group were two- to eightfold greater than those in group I during the first 4 weeks of infection. As with the production of antibody, the development of cellular immunity occurred more rapidly in group II mice. It required 19 days after infection before lymphocytes from group I animals were able to passively protect recipients against Karp challenge. In contrast, substantial resistance was transferable with spleen cells from group II mice only 11 days after infection. However, in each group the ability of donor mice to withstand potentially lethal challenge became apparent 1 to 2 days before demonstrable cell-transferable protection. This hiatus, between resistance of donor mice and their ability to passively transfer resistance with spleen cells, has been observed previously in this laboratory (10). It appears that certain maturation steps must take place in vivo, either of a qualitative or quantitative nature, before such cells can transfer immunity. In the same study (10) we noted that passive transfer of immune serum having a complement-fixing titer to Karp could protect a portion of the recipients against subsequent Karp challenge. Therefore, it is possible that serum antibody and cell-mediated immunity, both of which are below detectable levels during this hiatus period, can, in combination, protect donor mice against a potentially lethal challenge.

Several investigators have demonstrated the persistence of rickettsiae in tissues for long periods after successful treatment (4, 12, 14). An unexpected development of this study was finding large numbers of virulent rickettsiae associated with nonadherent, lymphocyte-enriched, spleen cells. When these cells were briefly homogenized to disrupt their physical integrity and physiological capabilities before transfer, fully lethal rickettsiae were easily demonstrated. Yet, the transfer of similar intact cells did not cause death of recipients but conferred protection on an immunologically naive host against subsequent exogenous challenge. Studies are currently in progress to identify the infected cell population and determine the effect of such parasitism on immune response development.

A delicate balance appears to be struck between immunization and proliferation. The marked difference in effectiveness of the group II treatment schedule suggests that immunization is dependent, in part, on rickettsial proliferation. A part of this dependence may be due to the accumulation of a critical antigenic mass. Indeed, the necessity for drug-free proliferation was suggested by previous experimental infections in mice (12) and studies in human scrub typhus (5, 8, 9, 11, 15), in which a short delay in initiation of antibiotic therapy after onset of symptoms resulted in protective immunity with fewer relapses. The temporary rickettsiostasis established with chloramphenicol seemed to preserve the balance between proliferation and immunization and supported previous data from this laboratory (1), which indicated that animals succumbing to Karp infection mount an immune response that is simply unable to keep pace with intracellular proliferation.

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