Role of Host Factors in the Pathogenesis of Salmonella-Associated Arthritis in Rats

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To evaluate the roles of the infectious agent and the host in the pathogenesis of Salmonella-associated arthritis (SAA), $10^3$ to $10^6$ heat-killed Salmonella enteritidis were injected directly into uninvolved carpal joints in normal and actively immunized rats and in hosts adoptively immunized by the intravenous transfer of spleen cells from syngeneic donors with ongoing SAA. As many as $10^6$ living Salmonellae invariably failed to generate more than a transient inflammatory response in normal rats. The regression of acute joint swelling was accelerated in both types of immunized hosts. The intensity and duration of acute inflammation evoked in normal rats by $10^6$ and $10^4$ heat-killed Salmonellae did not exceed the response elicited by $10^3$ living organisms. In sharply contrasting results, however, a chronic arthritis became established in a significant number of actively and adoptively sensitized rats after the intra-articular injection of $10^6$ heat-killed organisms. No Salmonellae were recovered from these adoptively sensitized rats although small numbers of organisms had been present among the spleen cells in the transfer inocula. Taken together, these results indicate the obligatory involvement of host factors in the mediation of this chronic arthritis and virtually eliminate any likelihood that joint damage in SAA is due to the directly destructive effects of intra-articular infection.

We reported in previous papers (5, 6) that a chronic destructive polyarthritis developed in rats subjected to an intravenous (i.v.) infection with Salmonella enteritidis. Certain features of the distribution and pathology of this Salmonella-associated arthritis (SAA) distinctly resemble human rheumatoid arthritis. The widely held belief that joint damage in rheumatoid arthritis is a consequence of an immunologically mediated attack leads to the logical corollary that is important to determine whether SAA is likewise due to an immunopathological process or merely the result of directly destructive effects of sustained intra-articular (i.a.) bacterial growth. Most of our evidence suggested that the joint damage in SAA is also immunologically mediated (5, 6). Two observations, however, were difficult to reconcile with this interpretation. One was that SAA arose only after the i.v. inoculation of living but not heat-killed (HK) Salmonellae. The second was that attempts to transfer SAA adoptively, even in the presence of specific antigen, were unsuccessful (6). The latter finding, however, is not compelling since SAA may not be wholly dependent upon a cell-mediated process. Weighing against the possibility that destructive infection per se could cause SAA was the failure to establish chronic arthritis in joints directly infected with living S. enteritidis (6).

To help clarify these apparent inconsistencies, we performed additional experiments involving the i.a. injection of living and HK S. enteritidis into normal rats, rats with ongoing SAA, and the recipients of adoptively transferred immune spleen cells. We found that in actively and adoptively immunized hosts the injection of HK S. enteritidis into a joint could lead to the development of a chronic arthritis.

MATERIALS AND METHODS

Animals. Young adult male (Lewis × BN) F1, hybrid (LBN) rats, 150 to 200 g, derived from highly inbred parents were conventionally maintained on a standard commercial diet and permitted tap water ad lib. The brood rats and stock from which the experimental rats were drawn were provided with drinking water containing oxytetracycline (Terramycin, Pfizer, New York, N.Y.) at a concentration of 65 mg/100 ml. Under prevailing conditions, a 200-g rat drinks about 50 ml/day. Treatment was discontinued 48 h before infection.

Microbial agents. S. enteritidis (NCTC 5694) was maintained under conditions described earlier (5). Challenge inocula were grown in tryptose soy broth (TSA, Difco, Detroit, Mich.) at 37°C for 6 h (late logarithmic growth phase), standardized turbidimetrically to approximately $10^6$ organisms per ml,
diluted suitably in saline, and immediately injected into a joint or tail vein. The number of viable bacteria in the challenge inoculum was assessed by plating suitably diluted saline suspensions on TSA and counting the number of colonies after overnight incubation at 37 C.

Intravenous infection. Rats were injected i.v. with $10^6$ to $3 \times 10^6$ viable *S. enteritidis*. The 50% lethal dose for 200-g LBN rats is about $5 \times 10^6$ viable organisms (5). In some experiments, *S. enteritidis* at 37 C. was killed by heating for 60 min at 60 C before injection.

**Intra-articular inoculation.** Suspending containing $10^5$ to $10^6$ living or dead organisms were injected into a grossly uninvolved carpal joint in a volume of 10 ul of pyrogen-free saline, using a 30-gauge needle mounted on a 50-μl syringe.

Cell suspensions. For the purpose of adoptive transfer, viable spleen cells prepared from donors with SAA were isolated according to methods previously described (7). The cells were suspended in phosphate-buffered saline, counted in a hemocytometer, and assessed for viability by means of eosin exclusion before being infused i.v. into normal adult recipients in numbers varying from 2 × $10^6$ to $4 \times 10^6$.

**Bacterial enumeration.** Quantitative bacterial counts were carried out on randomly selected groups of rats at appropriate time intervals after the i.v. injection of *S. enteritidis* (6). Saline homogenates of the whole spleen and the axillary lymph nodes were diluted suitably and plated directly onto TSA. The resulting colonies were suspended in nutrient broth and incubated at 37 C overnight for qualitative demonstration of bacteremias, whereas a 0.1-ml volume of blood was plated directly on TSA for quantitation when necessary. Aseptic technique was used to prepare cultures of whole joints that were exposed, excised, and ground in saline, using a sterilized pestle and mortar. The relative errors in the counting methods used were similar to those reported earlier (1, 3).

**DTH.** Delayed-type hypersensitivity (DTH) was assessed by injecting 5 μg of a *Salmonella* test antigen (2) in 0.1 ml of saline intradermally into the flank 5 to 20 days after infection. The increase in skin fold thickness after 3, 6, and 24 h was measured by means of a dial gauge caliper (1 unit = 0.1 mm) as previously described (4). The net increase was the measurement at the site of antigen injection minus the thickness of a saline-injected site on the opposite flank. A net increase of more than 9 units was significant at the 5% level (4). Measurements at 6 h never exceeded those at 3 or 24 h. Only the peak increases at 24 h are reported for the sake of brevity.

**Measurement of joint changes.** Joint pathology was scored as: 0, negative; 1, overt swelling but with contours of the joint maintained; 2, swelling with obliteration of contours; 3, gross distortion. The sum of the scores was taken as the joint swelling index for a panel of rats and adjusted to a maximum of 20 to facilitate comparison between panels containing different numbers of rats.

**RESULTS**

Effect of i.a. injection of living *S. enteritidis* on SAA. (i) Normal hosts. Figure 1 compares the development and regression of joint swelling among three panels of 19 rats after i.a. infection with $10^3$ to $10^6$ living organisms. It is clear that chronic arthritis did not develop in any of the rats thus treated. Twenty days after injection, the joint swellings had either completely receded ($10^6$) or were rapidly and clearly declining ($10^3$ to $10^5$). By 30 days, only an occasional rat in the latter group showed any residual inflammation, in each case judged to be trivial. In another experiment (not shown) even the i.a. injection of $10^6$ live *S. enteritidis* failed to induce persistent joint swelling. The failure of $10^3$ to $10^6$ organisms to induce chronic arthritis after their i.a. injection is highly significant in view of the consistent development of SAA that follows i.v. administration of these dosages of *Salmonella* (5, 6). To further evaluate the effects of i.a. infection, three rats at each dosage level were randomly selected on days 20 and 30 and skin tested for specific DTH. Measurements were read 3, 6, and 24 h later; 24-h increases are shown in Fig. 2. The rats were sacrificed immediately afterward, and samples of joints, liver, spleen, kidney, and brachial lymph nodes were examined for viable *Salmonella* (Fig. 2). Moderate to high degrees of 24-h skin test reactivity occurred in all the rats. Of the tissues later examined, bacteriologically positive cultures were obtained only from the brachial lymph nodes. Since some bacilli were found in the contralateral node, the systemic spread by the infection is inferred. Figure 2 shows little correlation between persistent

![Fig. 1. Intra-articular inoculation: live *Salmonella* in normal rats. The intensity and course of joint swelling developing after the injection of $10^3$ (A), $10^4$ (B), and $10^6$ (C) *S. enteritidis* directly into the left "wrist" in three panels of ten rats each. Three rats were removed from each panel on days 20 and 30 for sampling (See Fig. 2).](http://iai.asm.org/.../1155)
lymph node infection and the presence of residual joint swelling.

Local and disseminated bacterial growth resulting from the i.a. injection of living *S. enteritidis* was examined at more closely spaced intervals in an additional panel of rats. The time of onset and regression of joint swelling was wholly comparable with that shown for 10³ organisms in Fig. 1. The number of bacteria recovered and the 24-h skin test reactivity are recorded in Fig. 3. Initially, the number of viable *S. enteritidis* in the injected joint increased 10-fold before beginning to decline. Small numbers of viable *Salmonella* persisted in the joints for at least 10 days, but could not be recovered from any of the tested rats after day 20. The growth of *Salmonellae* in the liver and spleen was evanescent, and there was no evidence at any interval of bacterial lodgment in the kidneys. Small numbers of bacilli did, however, persist in the brachial lymph nodes for up to 30 days, at which time the experiment was terminated.

DTH in the i.a.-infected rats, shown as the net 24-h increase in flank skin thickness in Fig. 3, did not approach a maximum until day 10, in contrast to the much earlier (3 to 4 days) appearance of high levels of DTH observed in the i.v.-infected animals (5).

(ii) SAA-positive hosts. Figure 4 shows the results of i.a. inoculations of 10² and 10³ living *S. enteritidis*, respectively, into the grossly uninvolved joints of two groups of five rats with ongoing SAA of 1 month's duration. Severe swelling ensued in all of the injected joints almost immediately, but receded from the inoculated joints far more rapidly than was the case.
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in the naive hosts (Fig. 1). The resistance of the host under these conditions is clearly capable of effectively inactivating the injected organisms.

(iii) Recipients of spleen cells from SAA donors. Spleen cells were prepared from donors 10 to 12 days after i.v. infection with $5 \times 10^8$ S. enteritidis per 100 g of body weight (about 0.2 mean lethal dose). Approximately $4 \times 10^8$ viable spleen cells were injected i.v. into normal recipients on days 0, 4, and 7 of an i.a. infection with $10^9$ living S. enteritidis. The transfer of immune cells at all three intervals appeared to effect a more rapid decline in joint swelling when compared with control rats that were infused with comparable numbers of spleen cells taken from normal donors (Fig. 5).

Effect of the i.a. injection of heat-killed S. enteritidis in: (1) Normal hosts. Our past experience indicated that, in contrast to the intense acute inflammatory response that follows the i.a. injection of $10^8$ living S. enteritidis, only trivial inflammation is evoked by this number of dead organisms. To achieve joint swelling of comparable magnitude to that evoked by $10^8$ living S. enteritidis, (Fig. 1 and 4) it was necessary to inject $10^8$ HK S. enteritidis into the joint (Fig. 6). Bacterial cultures were routinely prepared from the inocula and from representative tissues as a precaution against accidental infection. Figure 6 also shows that the joint swelling that followed the i.a. administration of $10^8$ dead organisms reached maximum levels somewhat earlier and regressed more rapidly than when

FIG. 4. Intra-articular inoculation: live Salmonella in rats with SAA. The intensity and course of joint swelling developing after the injection of $10^3$ (●) and $10^6$ (○) live S. enteritidis directly into grossly uninvolved "wrists" of two panels of five rats with ongoing SAA.

FIG. 5. Intra-articular inoculation: live Salmonella in relation to adoptive transfer. The intensity and course of joint swelling developing in normal recipients after the injection of $10^3$ live S. enteritidis directly into the "wrists" in relation to the time of adoptive transfer of $4 \times 10^8$ spleen cells from syngeneic immunized (●) and normal (□) donors. The arrows indicate the time of i.v. infusion of donor cells.

FIG. 6. Intra-articular inoculation: dead Salmonella in normal rats. The intensity and course of joint swelling after the injection of $10^8$ (▲) and $10^6$ (●) heat-killed S. enteritidis directly into the "wrists" of normal rats. Each panel consisted of six rats.
10^6 and 10^4 live organisms were used (Fig. 1 and 3). Twenty-four-hour skin test reactivity to the Salmonella test antigens was found to be marginal, indicating that no significant DTH had been induced by the i.a. injection of HK Salmonella.

(ii) Hosts with ongoing SAA. Figure 7 compares the degree of joint swelling after the injection of 10^6 HK S. enteritidis into grossly uninvolved carpal joints in Salmonella-infected rats and normal controls. The infected rats had ongoing SAA of about 30 days' duration involving at least one joint. Although a statistical analysis of the data expressed in this way cannot readily be achieved, the curves shown in Fig. 7 make it clear that joint swelling in the SAA group after the i.a. injection of HK organisms was at every point more intense and persisted longer than in normal hosts. These results differ distinctly from the invariably evanescent joint swelling that follows the i.a. injection of live S. enteritidis into hosts with ongoing SAA (Fig. 4).

(iii) Recipients of spleen cells from donors with ongoing SAA. Suspensions of spleen cells were prepared from donors with a S. enteritidis infection of 10 to 20 days' duration and from normal rats. Each recipient was infused i.v. with approximately 4.5 x 10^6 viable spleen cells shortly before the i.a. injection of 10^6 or 10^4 HK S. enteritidis. The donor cells were washed three times by centrifugation at low rotational velocities (300 x g), but it was impossible to remove the live S. enteritidis from the cell inocula prepared from actively sensitized donors. As a result, about 6 x 10^6 viable Salmonellae were transferred in the 1-ml inoculum, a dosage that had previously been found to be ineffective for the induction of SAA in normal rats.

The i.a. injection of 10^6 but not 10^4 HK Salmonella, in the presence of spleen cells from donors with an emerging SAA, could lead to the development of a persistent arthritis in the recipients (Fig. 8). It is noteworthy that none of the rats receiving 10^6 HK S. enteritidis and transferred spleen cells developed persistent joint swelling, reinforcing the belief that the residual live S. enteritidis in the spleen cell suspension were not sufficient on their own to be arthritogenic. In the group that received 10^6 HK organisms, four out of six rats developed persistent joint changes; two were graded at 3+ reactions and two at 1+ by the end of this phase of the experiment on day 32. Cultures at this time of joints, liver, kidney, spleen, and brachial lymph nodes in the recipients of sensitized cells were uniformly negative. Control rats injected i.a. with 10^6 dead Salmonellae and i.v. with normal spleen cells did not develop persistent joint swelling.

(iii) Rechallenge with living S. enteritidis i.v. Initially normal rats that had been injected i.a. with either living or dead Salmonellae were given a secondary i.v. challenge of 5 x 10^6 living S. enteritidis per 100 g of body weight about 35 to 37 days after primary inoculation. In no instance did joint lesions appear over the 30 additional days of observation. In another experiment, four groups of ten rats were injected i.a., respectively, with 100 µg of one of the following agents, each present in 0.01 ml of saline: bovine serum albumin, keyhole limpet hemocyanin, HK S. enteritidis whole cells, and S. enteritidis purified lipopolysaccharide. Figure 9 shows a comparison of the intensity and

![Fig. 7. Intra-articular inoculation: dead Salmonella in rats with SAA. The intensity and course of joint swelling after the injection of 10^6 (△), and 10^4 (○) heat-killed S. enteritidis directly into uninvolved "wrists" in rats with ongoing SAA (○) and in normal controls (○).](image-url)
duration of the joint swelling resulting from each treatment. In no instance did the initial swelling persist beyond 8 days. On day 14, the rats in each group were infected i.v. with $5 \times 10^6$ live \textit{S. enteritidis} per 100 g of body weight and observed regularly for the appearance of SAA. Twenty-one days later, six of ten rats that had received primary inoculations of the purified proteins before infection were displaying arthritis in one or more joints. Joint swelling was still present 3 weeks later when observation was terminated. No joint lesions were observed, however, in the two groups of rats that had been previously treated with HK whole cells or lipopolysaccharide.

**DISCUSSION**

Since chronicity, taken as the persistence of joint swelling in excess of 28 days (5), is one of the most characteristic features of i.v.-induced SAA, this criterion was applied in the present study. From the data presented in this report, it is clear that infecting a joint directly with up to $10^4$ living \textit{S. enteritidis} does not induce a chronic arthritis either in naive or in actively or adoptively sensitized rats. The $10^8$ live organisms injected i.a. is undoubtedly a realistic quantity in the sense that comparable numbers of \textit{S. enteritidis} can be recovered from infected joints in rats inoculated i.v. with $10^8$ organisms (6). The i.a. inoculation of living \textit{Salmonellae} into actively immunized rats produces an even more transient joint swelling than in naive ani-

mals (Fig. 1 and 4). In adoptively immunized hosts there is a demonstrable correlation between the accelerated decline of joint swelling and the time of spleen cell transfer (Fig. 5). Other experimental data suggest indirectly that this effect is specific since heterologous antigens injected i.a. do not protect against the subsequent development of SAA after an i.v. rechallenge with live \textit{S. enteritidis}, but homologous antigens do.

In distinct contrast to the transient inflammation invariably observed after the i.a. injection of living organisms, the injection of $10^8$ HK \textit{S. enteritidis} by this route induces a chronic arthritis in a significant number of actively or adoptively immunized hosts. This finding is not paradoxical when considered in terms of the local antigenic load likely to accumulate in the joint during these procedures. For example, the data in Fig. 3 show that live \textit{S. enteritidis} are effectively inactivated within the injected joints as well as at sites of systemic dissemination. On the other hand, after the i.v. administration of live \textit{S. enteritidis} the prolonged bacteremia and the ensuing carrier state (5) undoubtedly provide greater opportunity for the organisms to penetrate the joints repeatedly and effect the accumulation of antigen. Since it is plausible that i.v.-injected dead organisms cannot gain access to the joint, the apparent dependence of the development of SAA on live organisms (6) may therefore indicate a requirement for antigenic buildup rather than for articular infection. Theoretically, a large i.a. inoculum of living organisms should be as effective in the evolution of chronic arthritis as $\times 10^9$ HK \textit{Salmonellae}. In practice, this cannot be tested since the injection of $10^9$ living \textit{Salmonellae} into the joint results in extensive systemic infection with resultant multifocal arthritis (unpublished data). Dead organisms, on the other hand, can be injected into the joint in very large numbers, establishing antigenic accumulations that may not be readily removable.

Taken together, the foregoing observations underscore the importance of the response of the host as an obligatory factor in the development of SAA. In addition, development of chronic arthritis in actively or adoptively immunized but not normal rats after the i.a. injection of HK \textit{Salmonellae} supports our belief that SAA is an immunologically mediated process. As yet, the extent to which cellular and humoral factors participate in the pathogenesis of this syndrome cannot be determined. Data in some experiments suggest cellular mediation; they are, however, also consistent with a humoraly mediated process. Indeed, studies cur-
rently in progress suggest that antibody may well be a determinant in the pathogenesis of SAA.

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LITERATURE CITED


