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H-2 Restriction in Acquired Cell-Mediated Immunity to Infection with *Francisella tularensis* LVS

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The *H-2* restriction imposed on the T-lymphocyte–macrophage interaction leading to the expression of acquired cellular immunity was evaluated in an experimental model of infection with the live vaccine strain of *Francisella tularensis*. Restriction between T cells and macrophages was examined in vitro in cultures containing macrophages from C57BL/10 (B10) mice, T cells from immune B10 *H-2* congenic mice, and *F. tularensis* antigen. The cellular interaction was assayed by the production in the cultures of factors which stimulate thymocyte DNA synthesis. It was observed that homology at the I-A region of the *H-2* complex was required for productive T-cell–macrophage cooperation to occur. Restriction was also investigated in an in vivo passive cell transfer system. Spleen cells from immunized B10 mice were injected into naive B10 *H-2* congenic mice, which were then challenged with *F. tularensis*. Enhanced resistance to the challenge infection in recipient mice was used as a marker of a successful T-cell –macrophage interaction. It was found that when the recipient strain shared H-2 I-A region homology with the donor strain, enhanced antitularemic resistance was expressed, whereas homology at the *H-2 K* or *D* region was insufficient. Thus, macrophage–T-cell cooperation in immunity to experimental tularemia was restricted at the level of class II determinants.

In the early 1970s, it was discovered that intercellular recognition in acquired immune responses required genetic homology between the cells with respect to major histocompatibility complex loci (21, 24). Since then, studies of the phenomenon of *H-2* restriction have led to a deeper understanding of the mechanisms by which cells of the immune system interact to perform their functions. Indeed, studies of cellular interactions and *H-2* restriction elements have promoted the general concept that the type of cellular interaction, i.e., helper or cytotoxic, is directly correlated with the class of the *H-2* element which restricts it (reviewed in reference 22). Thus, examination of this phenomenon is instructive in the elucidation of the mechanisms of cell-mediated immunity.

One type of immune response which has attracted a great deal of attention is that which controls infection with facultative intracellular pathogens. In the last decade, attention has been directed toward the *H-2* restriction imposed upon the cellular interactions leading to the expression of acquired resistance to these organisms. Although many experimental models of intracellular bacterial infections exist, major histocompatibility complex restriction has been examined in only one, namely, that of murine listeriosis. In this model, however, confusion has arisen with respect to the identity of the relevant *H-2* restriction element; this element has been variously reported to be a class I (6, 18) or a class II (9, 12, 23) gene product. It was, therefore, of great interest to investigate the phenomenon of *H-2* restriction in another experimental model. We have established a model of experimental tularemia that uses the live vaccine strain of *Francisella tularensis*. The resolution of infection with this facultative intracellular bacterium in mice is known from previous studies to require T cells (1; L. S. D. Anthony and P. A. L. Kongshavn, submitted for publication), of which the $L3T4^+$ subpopulation exhibits the predominant, but not exclusive, activity. The nature of the *H-2* restriction element in the response to tularemia has never been evaluated; in the present investigation, we have undertaken to determine the class of *H-2* molecule which restricts the T-cell–macrophage interaction leading to the expression of antitularemic resistance.

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MATERIALS AND METHODS

Mice. Adult male or female mice of strains C57BL/10 (B10), B10.BR, B10.A(2R), B10.A(5R), and B10.MBR were purchased from Jackson Laboratory, Bar Harbor, Maine. C57BL/10Sn (B10/Sn) and B10.D2 mice were bred in our laboratory from breeding stock obtained from Jackson Laboratory. B10.STA62 mice were bred in our facilities from stock generously provided by Jan Klein of the Max-Planck Institut für Biologie, Tübingen, Federal Republic of Germany. All mice were housed under conventional conditions and given food and water ad libitum.

Bacteria. *F. tularensis* LVS was obtained from the American Type Culture Collection, Rockville, Md. Bacteria were grown to a density of approximately 2×10^8 CFU/ml in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and stored frozen in aliquots at −80°C. For each experiment, an aliquot was thawed and diluted in sterile normal saline to an appropriate concentration for injection. The actual inoculum was verified retrospectively by plating a suitable dilution of the stock on cystine heart agar (Difco Laboratories, Detroit, Mich.) containing 5% defibrinated rabbit blood (Institute Armand-Frappier, Laval, Quebec, Canada).

In vitro assay of T-cell–macrophage cooperation. The interaction between T cells and macrophages was assessed in vitro by measuring the production of thymocyte mitogenic factors (TMF) in cultures of macrophages, *F. tularensis*-immune lymphocytes, and heat-killed *F. tularensis* antigen (HKFT) essentially by the procedure of Farr et al. (9). Briefly, lymphocyte donor mice were immunized by an

intravenous injection of approximately 100 CFU of *F. tularensis*. After 11 days, these mice, together with unimmunized normal macrophage donor mice, were injected intraperitoneally with 1.5 ml of 10% proteose peptone broth (Difco). Three days later, peritoneal exudate cells were collected from both groups of mice by peritoneal lavage, and the immune lymphocyte peritoneal exudate cells were enriched for T lymphocytes by nylon wool adherence (11). Flat-bottomed, 96-well culture dishes were seeded with 4×10^4 macrophages, 4×10^4 immune lymphocyte peritoneal exudate cells enriched for T lymphocytes, and 4×10^6 HKFT suspended in RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and antibiotics. The cultures were incubated in triplicate for 20 to 24 h at 37°C in a humidified atmosphere containing 5% CO_2. Supernatants from identical triplicate wells were collected, pooled, and assayed for TMF. To accomplish this, we seeded 96-well dishes in triplicate with 1.25×10^6 A/J mouse thymocytes suspended in RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and antibiotics and containing a 25% (vol/vol) concentration of test supernatant. After approximately 42 h of incubation, each well was pulsed with 1 μCi of tritiated thymidine (6.7 Ci/mmol; ICN Biomedicals, Montreal, Quebec, Canada) and incubated for a further 6 h. The DNA was extracted on glass fiber filters, which were then suspended in scintillation fluid and counted in a beta emission counter. Results are expressed as counts per minute of incorporated radiolabel.

In vivo assay of T-cell–macrophage cooperation. Donor mice of the B10 and B10/Sn strains were immunized with 100 CFU of *F. tularensis*. Single cell suspensions were prepared from donor spleens and passed over nylon wool. Nonadherent spleen cells were washed and injected into naive recipient mice at a dose of 50×10^6 viable cells per mouse. Control mice received 50×10^6 spleen cells from normal, uninfected B10 and B10/Sn mice. All recipient mice were then challenged with an intravenous inoculum of approximately 10^5 CFU. The number of viable bacteria present in the spleen cell inoculum (typically 50 to 100 CFU) was considered to be insignificant in comparison with the magnitude of the challenge inoculum. After 3 days, the mice were sacrificed and the bacteria in the spleens were enumerated by previously described methods (1). In this in vivo system, the cell recipient is the source of effector macrophages, while the cell donor provides immune T cells.

RESULTS

Selection of congenic mouse strains. Congenic mouse strains selected for conducting the *H-2* restriction experiments were those which differed at one or more than one defined region of the mouse major histocompatibility complex-encoding class I (*K* or *D*) or class II (*I-A*) antigenic determinants. Since it was desirable to avoid effects caused by the influence of heterologous background genes which differ among various mouse strains (3, 5), a series of *H-2* congenic mouse strains with a common B10 background was chosen, and the haplotypes of these strains are described in Table 1 (17).

***H-2* restriction measured in vitro.** The cooperation between T cells and macrophages in antitularemic resistance was first evaluated in a culture system with elicited peritoneal macrophages from unimmunized mice, T-cell-enriched peritoneal exudate cells from immunized mice, and HKFT as antigen. When antigen-specific T cells and antigen-presenting macrophages interact in this system, lymphokines and monokines (9, 10, 13), including TMF (9), are produced. Therefore, the presence of TMF in these cultures is an index of the ability of T cells and macrophages to cooperate successfully. Figure 1 shows the results of the assay for TMF activity when macrophages from B10 mice and immune T cells from B10 (*H-2*^{b}) or B10 congenic mice were cultured together in the presence of antigen. The supernatant from control cultures containing only macrophages and HKFT resulted in a background tritiated thymidine uptake of approximately 4,000 cpm. Cultures containing B10 T cells, which are homologous with B10 macrophages at all *H-2* loci, contained a concentration of TMF sufficient to result in a thymocyte tritiated thymidine incorporation of about 13,000. In contrast, cultures containing B10 macrophages and B10.D2 (*H-2*^{d}) T cells, a combination with no *H-2* homology, produced only background levels of TMF. Homology at the *D* region was insufficient for a successful T-cell–macrophage interaction, as evidenced by the lack of TMF production in cultures containing B10.A(2R) immune T cells. The production of TMF was observed when T cells were homologous with macrophages at the *K* and *I* regions [the B10.A(5R) strain]. However, since *K* region homology between T cells and macrophages was by itself insufficient for the induction of TMF activity, as evidenced by the B10-B10.MBR strain combination, it can be concluded that *I* region homology is necessary and sufficient for macrophage–T-cell interactions in vitro. Since the *I-E* region-encoded molecule is not expressed in B10 mice (19), the necessary homology is at the level of the *I-A* region-encoded molecule.

TABLE 1. *H-2* haplotypes of B10 congenic strains used in this study[a]

Mouse strain	*H-2* haplotype	Alleles at *H-2* loci							
		K	*I-*				*S*	*D*	*L*
			A_β	A_α	E_β	E_α			
B10	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
B10.D2	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*
B10.BR	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*
B10.A(2R)	*h2*	*k*	*k*	*k*	*k*	*k*	*d*	*b*	*b*
B10.A(5R)	*i5*	*b*	*b*	*b*	*b*	*k*	*d*	*d*	*d*
B10.MBR	*bq1*	*b*	*k*	*k*	*k*	*k*	*k*	*q*	*q*
B10.STA62	*w27*	*w27*	*b*	*b*	*w27*	*w27*	*w27*	*w27*	*w27*

[a] Adapted from Klein et al. (17).

***H-2* restriction measured in vivo.** In the next series of experiments, the restriction element for T-cell–macrophage interactions in antitularemic immunity was evaluated in an in vivo model of passive transfer of resistance by immune lymphocytes. These experiments were designed such that the cell recipient would be the source of macrophages, while the cell donor would provide the *F. tularensis*-sensitized lymphocytes. In this in vivo system, effective cooperation between T cells and macrophages leads to the expression of antitularemic immunity, which is reflected by the accelerated clearance of a challenge infection. The results of two similar experiments in which B10 congenic mice were the recipients of donor cells from immunized B10 mice are shown in Table 2. Transfer of immune B10 cells into syngeneic B10 recipients conferred a $\log_{10}$ protection against the growth of bacteria in the challenge inoculum of 1.6 to 1.9. In contrast, allogeneic B10.BR recipients were not protected to a greater extent by immune B10 cells than by normal B10 cells. Among the other B10 congenic mice, a $\log_{10}$ protection of up to 1.5 was observed in the B10.A(5R) and B10.STA62 mice (homology with *H-2*^{b} at *H-2 K* and *I-A* and at *H-2 I-A*

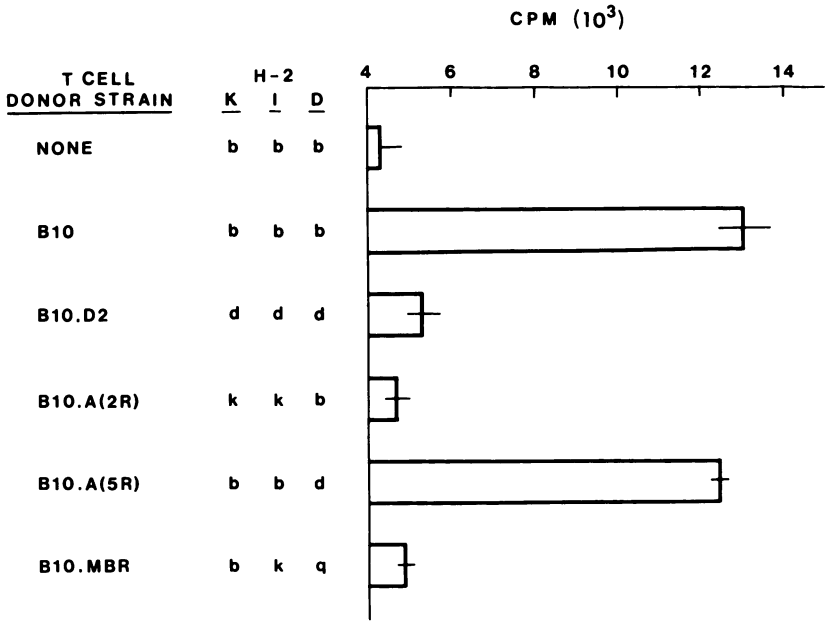

FIG. 1. *H-2* restriction of macrophage–T-cell interactions as measured in vitro. Proteose peptone-elicited peritoneal macrophages from unimmunized B10 mice were cultured with HKFT and nylon wool-purified peritoneal lymphocytes from immunized, proteose peptone-treated B10 or B10 congenic mice. Culture supernatants were assayed for TMF activity on A/J strain thymocytes. Each bar represents the mean and standard error of the mean counts per minute of tritiated thymidine incorporated into triplicate thymocyte cultures. Control wells were macrophages cultured with HKFT and no T cells.

alone, respectively). B10.BR recipients of normal B10 cells did not demonstrate enhanced antibacterial resistance (Table 2, experiment 1), indicating that allogeneic rejection phenomena alone did not increase antitularemic resistance. The multiplication of *F. tularensis* in B10.A(2R) and B10.MBR recipients was similar to that observed in allogeneic B10.BR recipients. Thus, the successful cooperation between T cells and macrophages, as measured by a passively acquired state of enhanced antibacterial resistance, is dependent upon genetic homology at the *I-A* region of the *H-2* complex.

DISCUSSION

In the experiments described in this report, *H-2* restriction between T lymphocytes and macrophages was examined in an experimental model of resistance to infection with *F. tularensis* LVS. The results of these experiments demonstrated clearly that the cellular interactions leading to expression of antitularemic immunity are restricted by homology at *H-2* loci coding for class II (*I-A*) determinants, whether these interactions are measured in vitro (Fig. 1) or

TABLE 2. *H-2* restriction of the expression of passively acquired resistance to tularemia

Expt	Donor[a]	Recipient	Homologous *H-2* region(s)	Log_{10} CFU of *F. tularensis*/spleen[b]	Log_{10} protection[c]
1	B10 normal	B10	*K, I, D*	6.94 ± 0.06	
	B10 normal	B10.BR	None	7.18 ± 0.02	−0.24
	B10 immune	B10	*K, I, D*	5.32 ± 0.10	1.62
	B10 immune	B10.BR	None	6.65 ± 0.19	0.29
	B10 immune	B10.A(2R)	*D*	6.57 ± 0.06	0.37
	B10 immune	B10.A (5R)	*K, I*	5.47 ± 0.14	1.47
	B10 immune	B10.MBR	*K*	6.68 ± 0.06	0.27
	B10 immune	B10.STA62	*I*	5.40 ± 0.14	1.54
2	B10 normal	B10	*K, I, D*	6.84 ± 0.11	
	B10 immune	B10	*K, I, D*	4.92 ± 0.13	1.92
	B10 immune	B10.BR	None	6.35 ± 0.08	0.49
	B10 immune	B10.A(2R)	*D*	6.28 ± 0.07	0.56
	B10 immune	B10.A(5R)	*K, I*	5.40 ± 0.10	1.44
	B10 immune	B10.MBR	*K*	6.35 ± 0.09	0.49
	B10 immune	B10.STA62	*I*	5.65 ± 0.19	1.19

[a] Normal B10 cells were 50×10^6 untreated spleen cells from uninfected B10 mice, and immune B10 cells were 50×10^6 nylon wool-enriched spleen cells from B10 mice immunized 7 days previously by injection with about 100 CFU of *F. tularensis*.

[b] Mean ± standard error of the mean log_{10} CFU of *F. tularensis* isolated from the spleens of three to six mice injected 3 days previously with approximately 10^5 CFU.

[c] Log_{10} protection = log_{10} CFU of *F. tularensis* per spleen of recipients of normal B10 cells − log_{10} CFU of *F. tularensis* per spleen of recipients of immune B10 cells.

in vivo (Table 2). These observations were made in a series of *H-2* congenic strains with a common B10 background genome. Therefore, the failure of immune B10 cells to transfer protection to B10.A(2R) or B10.MBR mice was probably not due to restriction occurring across non-*H-2* gene barriers, a phenomenon observed by others (3, 5). Neither do we consider allogeneic rejection reactions, caused by *H-2* dissimilarities between lymphoid cell donors and cell recipients, to have significantly affected the results of the passive cell transfer experiments, because the period between the time of cell transfer and the time of sacrifice was only 3 days. In addition, mice experiencing strong allogeneic reactions, for example, early in the course of graft-versus-host disease, actually demonstrate enhanced antibacterial resistance (2, 4, 20). We observed, however, a very slight decrease in resistance to *F. tularensis* infection in B10.BR recipients of spleen cells from unimmunized B10 mice (Table 2, experiment 1), indicating that no such allogeneic effects occur in this system.

The results of our experiments with *F. tularensis* are in agreement with those obtained by other investigators working with *Listeria monocytogenes* infections in vivo (23) and in vitro (9). On the other hand, our observations are in opposition with those made in more recent studies of listeriosis, in which the passive transfer of resistance (6) and granuloma formation (18) are restricted by class I (*H-2 K* region-encoded) determinant homology. This discrepancy is as yet unexplained. However, it is unlikely that it is due to major differences in experimental procedures, since our cell transfer experiments were performed in a manner similar to those of Cheers and Sandrin (6) with respect to the source of immune cells (i.e., spleen) and the use of congenic mouse strains with a common genetic background. It is now well accepted that restriction by class I determinants is generally associated with interactions involving Lyt-2^+ (cytotoxic) cells, whereas class II determinants generally restrict interactions mediated by L3T4^+ (helper) cells (22). The difference between tularemia and listeriosis with respect to restriction elements thus probably reflects differences in the nature of the cellular response to these infections. Indeed, we have shown that the expression of antitularemic resistance depends mainly upon the activity of a population of L3T4^+ cells, with the Lyt-2^+ cell population playing a minor role (unpublished observation). In contrast, Näher and coworkers have found that the most effective resistance to *L. monocytogenes* infection is associated with the formation of granulomas in the tissues and that granuloma formation is dependent upon Lyt-2^+ cells (18).

One can speculate that the resolution of infection with intracellular bacteria involves both L3T4^+ (helper) and Lyt-2^+ (cytotoxic) cell populations acting at different phases of bacterial elimination. Thus, helper T cells may be necessary early in the course of elimination to initiate macrophage activation via macrophage-activating factors and/or gamma interferon or perhaps to lyse infected macrophages (15). At this time also, they may promote the expansion of Lyt-2^+ cytolytic effector cells through the secretion of interleukin-2. The latter cells may act later by (i) replacing L3T4^+ cells in the induction of lymphokine-mediated macrophage activation, since they have been shown to release gamma interferon (7, 8, 14), and/or (ii) lysing infected macrophages, as has been demonstrated in vitro for macrophages infected with *L. monocytogenes* (8, 14), *Mycobacterium bovis* BCG (8), or *Mycobacterium leprae* (7). The cell population seen to transfer resistance might then depend upon the time during the elimination phase at which the cells were collected for transfer. The class of restriction element observed would in turn depend upon the type of cell (L3T4^+ or Lyt-2^+) most active at that time. For example, in our model of tularemia, class II restriction was observed because the immune cells were collected for transfer at a time when the L3T4^+ cell population was critical for bacterial elimination. In a study by Zinkernagel et al., who also demonstrated class II restriction, the transferred cells may have been collected at the same relative time during the course of elimination of *L. monocytogenes* (23). In listeriosis studies in which class I restriction was observed, the cells may have been collected after the Lyt-2^+ cell population had expanded and taken over the burden of bacterial elimination hitherto borne by the L3T4^+ cell population. The finding of Näher et al. that class I-restricted Lyt-2^+ cells were more effective at transferring granuloma formation/resistance than were Lyt-1^+ cells supports this interpretation (18). It would be interesting, therefore, to test this hypothesis by monitoring the *H-2* restriction phenomenon throughout the course of the host response to tularemia to determine whether the restriction element shifted from class II to class I and to determine whether the cell population critical for the adoptive transfer of resistance shifted from L3T4^+ cells to Lyt-2^+ cells. It would also be interesting to determine the restriction element(s) in the experimental model of Kaufmann et al., in whose experiments the passive transfer of antilisterial resistance required the participation of both the L3T4^+ and Lyt-2^+ cell populations (16).

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