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REQUIREMENT FOR CONTINUOUS ANTIGENIC STIMULATION OF IMMUNE SPLE--ETC(U)
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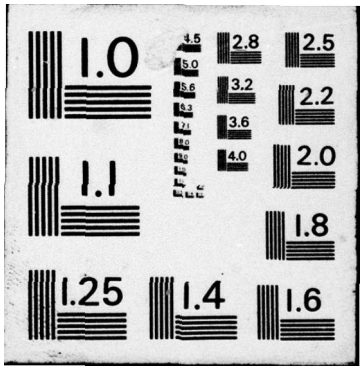
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REQUIREMENT FOR CONTINUOUS ANTIGENIC STIMULATION OF IMMUNE SPLEEN CELLS IN ADOPTIVE TRANSFER OF CELL-MEDICATED PROTECTION AGAINST TULAREMIA

ARMY MEDICAL RESEARCH INSTITUTE OF
INFECTIOUS DISEASES, FREDERICK, MARYLAND

14 DECEMBER 1976

Requirement for Continuous Antigenic Stimulation of Immune Spleen Cells in Adoptive Transfer of Cell-mediated Protection against Tularemia

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Running Head: ANTIGENIC REQUIREMENT FOR TRANSFER OF CMI

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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ABSTRACT

Under appropriate conditions passively transferred spleen cells from AKR/J mice immunized with live Francisella tularensis confer high-grade protection (survival approaching 100%) to nonimmune syngeneic recipients against intravenous, intraperitoneal, or subcutaneous challenge with a fully virulent strain. When experimental conditions were adjusted to eliminate viable cell-associated F. tularensis, adoptive immunity was abrogated. However, concurrent administration of appropriate concentrations of homologous killed organisms and immunocompetent spleen cells devoid of viable organisms provided conditions for restoration or enhancement of protective activity; recipients treated in this manner were resistant to infection for at least 47 days. Short-term nonspecific protection against intraperitoneal but not against subcutaneous challenge was elicited by injection of immune cell recipients with appropriate concentrations of killed organisms from taxonomically unrelated genera. In this model system, complicating effects introduced by transfer of replicating organisms have been eliminated and survival can be used to evaluate protection.

In a recent study on cell-mediated immunity we described an experimental murine model in which passive transfer of spleen (lymphoid) cells from immunized donors enables syngeneic recipients to survive an otherwise fatal infection with fully virulent Francisella tularensis SCHU S5 (10). Although viable cell-associated F. tularensis in transferred donor cells was not a significant factor in the protection of immune spleen cell (ISC) recipients, a definitive model required the elimination of those organisms capable of replication from preparations of donor immunocompetent cells. However, "immune" spleen cells devoid of viable cell-associated microorganisms, either by virtue of a prolonged interval between donor immunization and transfer or as a result of booster inoculation with an attenuated rather than a fully virulent strain, failed to protect recipients against fatal infection after challenge (10). It was hypothesized, therefore, that immunologically committed spleen cells required a specific antigenic stimulus to ensure sufficient activation of macrophages for expression of immunity (25).

The present study, confirming and expanding these observations, demonstrates that recent activation of sensitized cells by a specific antigenic stimulus is necessary for the adoptive transfer of protection against a highly virulent challenge.

MATERIALS AND METHODS

Animals. Inbred male and female AKR/J mice (Jackson Laboratories, Bar Harbor, ME) were maintained in our holding facilities for 4-6 weeks before experimentation. Immunization of donors was initiated when mice were 12 to 14 weeks of age. Syngeneic recipients of transferred spleen cells were 12 to 14 weeks of age and always of the same sex as the donors.

F. tularensis strains and cultures. Lyophilized attenuated live tularemia vaccine (9) prepared from strain LVS (Merrill-National Laboratories, Philadelphia, PA) and stored at -20 C was rehydrated with sterile water for injection and thoroughly mixed by passage through a 22-gauge needle. The two fully virulent F. tularensis strains, SCHU S4 and its streptomycin-resistant mutant SCHU S5, are identical in every respect with the exception of antibiotic sensitivity, and are equally capable of causing severe, potentially fatal disease in humans or death in various laboratory animals infected with as few as 1 to 10 organisms (26-29). These strains were cultivated in modified casein partial hydrolysate liquid medium (MCPH) (9) and aliquots of each culture were stored at -60 C for 1 to 6 months; frozen cultures were thawed at 37 C immediately before use. Periodically, virulence assays were performed in rabbits to ensure that subcutaneous (s.c.) injection of 5 to 10 organisms of either SCHU S4 or SCHU S5 would result in death within 10 days. The viable population in cultures was estimated after dilution in sterile gelatin-saline (0.1% gelatin and 0.9% NaCl); 0.2-ml aliquots were spread on the surface of glucose-cysteine-blood agar, and typical colonies were enumerated after incubation at 37 C for 48 to 72 h.

Nonviable antigens. MCPH cultures of F. tularensis SCHU S4, 30 x 10^9 viable organisms per ml, were killed with 0.5% phenol. Plague vaccine U.S.P. (Cutter Laboratories, Inc., Berkeley, CA) contained formalin-killed Yersinia pestis organisms adjusted to a concentration of 2 x 10^9/ml. Each antigen was centrifuged at 16000 x g for 30 min at 5 C, washed 3 X in sterile physiological saline solution (PSS), and resuspended to the appropriate concentration with PSS. Antigen was administered intraperitoneally (i.p.) in

0.2-ml amounts at the time of spleen cell transfer.

Immunization. Male mice were used for the preliminary study (Table 1) whereas female mice were used in all subsequent experiments. Immunization of male mice with live tularemia vaccine (strain LVS) was comparable to that previously described (10): 10^2 LVS organisms were administered s.c., followed 30 $\pm$ 2 days later by an i.p. booster dose of viable organisms, 10^6 LVS or 10^3 SCHU S4. Female mice were immunized s.c. with 10^3 LVS organisms because they were more resistant to infection with the attenuated strain. Usually 5 to 15% of the male mice, but only 1 to 6% of female mice, succumbed after initial inoculation with 10^2 LVS organisms; following the SCHU S4 booster dose, 70 to 90% of LVS-immune males survived as compared to 61 to 80% of corresponding female mice. When 10^3 LVS were administered initially to female mice, 6 to 9% succumbed to the immunizing dose and 69 to 94% survived the SCHU S4 booster.

Spleen cell suspensions and measurement of resistance. Spleen cell suspensions were prepared and adoptive transfers were performed as previously described (10). Spleens were minced with sterile scalpels and pressed through sterile 60-mesh stainless-steel screens (Small Parts Co., Miami, FL) into cold RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 100 U of penicillin and 100 µg of streptomycin per ml. Cell clumps were dispersed by gentle agitation and passage through a 25-gauge needle. Suspensions were centrifuged at 250 x g for 10 min at 5 C, washed twice with antibiotic-free cold RPMI, and resuspended in the same menstruum. Spleen cell suspensions contained 95 to 99% viable cells when tested by trypan blue (0.5%) exclusion.

Recipients were injected i.p. with 1 ml of spleen cell suspension within 1 h after preparation. At least 1 ml of each suspension was used to

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Under appropriate conditions passively transferred spleen cells from AKR/J mice immunized with live Francisella tularensis confer high-grade protection (survival approaching 100%) to nonimmune syngeneic recipients against intravenous, intraperitoneal, or subcutaneous challenge with a fully virulent strain. When experimental conditions were adjusted to eliminate viable cell-associated F. tularensis, adoptive immunity was abrogated. However, concurrent administration of appropriate concentrations of homologous killed organisms and

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immunocompetent spleen cells devoid of viable organisms provided conditions for restoration or enhancement of protective activity; recipients treated in this manner were resistant to infection for at least 47 days. Short-term non-specific protection against intraperitoneal but not against subcutaneous challenge was elicited by injection of immune cell recipients with appropriate concentrations of killed organisms from taxonomically unrelated genera. In this model system, complicating effects introduced by transfer of replicating organisms have been eliminated and survival can be used to evaluate protection.

estimate the number of spleen cell-associated microorganisms; aliquots of the suspension or dilutions in gelatin-saline were spread on the surface of glucose-cysteine-blood agar medium, and colonies were counted after 48 to 72 h of incubation.

Syngeneic recipients were challenged i.p. or s.c. with 25 to 50 organisms, 25 to 50 minimal lethal doses (MLD), of streptomycin-resistant strain SCHU S5 at various intervals (1 to 30 days) after spleen cell transfer. Survivors/total (S/T) were recorded at 20 days post-challenge. When transfer of viable ISC-associated organisms was anticipated, treatment with streptomycin (400 μg in 0.1 ml administered s.c. two or three times daily) was initiated immediately after transfer to eliminate or suppress extracellular growth of cell-associated streptomycin-sensitive LVS or SCHU S4. Control groups with normal AKR/J mice or recipients of normal syngeneic spleen cells were treated in the same manner to ensure the validity of the response to challenge with the streptomycin-resistant SCHU S5 strain. No significant differences in mean time to death were demonstrated between these control groups. Thirty days following ISC transfer, recipients that survived a SCHU S5 challenge at 1 to 14 days after transfer as well as groups of nonchallenged recipients were injected s.c. with 10^2 MLD (100 to 150 organisms) of streptomycin-sensitive strain SCHU S4, and survival was recorded for 15 days. Control groups of AKR/J mice were included with every challenge: (a) mice that received no pretreatment, (b) recipients of 1×10^8 normal spleen cells (NSC), (c) recipients of NSC concurrently with nonviable antigen, or (d) mice injected with killed antigen alone.

RESULTS

Protective activity of immune spleen cells from streptomycin-treated donors. Male AKR/J mice were immunized s.c. with 10^2 viable LVS and 30-day survivors were administered an i.p. booster of 10^3 viable SCHU S4 organisms. At various times after the booster dose, comparable groups of mice were treated with 400 µg of streptomycin every 8 h for 3 to 6 consecutive days. Twleve days post-booster the mice were sacrificed and their spleens were processed for ISC suspensions; 1 x 10^8 ISC were transferred to syngeneic recipients which were challenged i.p. 20 h later with streptomycin-resistant strain SCHU S5.

Streptomycin treatment administered to donors after a booster injection significantly reduced the number of viable SCHU S4 organisms associated with donor splenocytes (Table 1). Moreover, the capability of ISC from treated donors to confer adoptive immunity on recipients was also reduced appreciably; regardless of the treatment schedule employed for donors, only 3 of 30 recipients survived challenge whereas 4 of 6 streptomycin-recipients of ISC from nontreated donors survived.

In another experiment, ISC harvested at various times (4 to 47 days post booster) from LVS-immunized SCHU S4-boostered donors were transferred to syngeneic recipients (1 donor spleen/recipient), and recipients were challenged 20 h later (Table 2). In contrast to the complete protection afforded recipients by ISC transferred 4 and 8 days post-booster, no protection was provided by ISC 6-day post-booster, possibly because the number of spleen cell-associated SCHU S4 (10^6 viable organisms) constituted too formidable a challenge despite streptomycin therapy. The 10- to 18-day ISC contained decreasing numbers of SCHU S4 organisms and conferred practically complete

resistance, while 21-, 35- or 45-day post-booster ISC were essentially devoid of live spleen cell-associated SCHU S4 and recipients evidenced decreased protection, i.e., 60, 40 or 7% survival, respectively. At 47 days post-booster ISC were ineffective unless 10^7 killed SCHU S4 antigen was given concurrently (7 of 8 mice survived challenge). All control mice died within 11 days. These data support the hypothesis that a minimal amount of antigen is required for expression of immune competence by transferred spleen cells.

Effect of killed F. tularensis antigen on adoptive protection afforded recipients of syngeneic spleen cells. In our previous studies (10), ISC suspensions from LVS-boostered donors were free of viable spleen cell-associated LVS organisms and failed to transfer protection to syngeneic recipients. Consequently, the LVS-boostered donor mouse appeared to be an excellent model for investigating the contribution of nonviable specific antigen to adoptive transfer of resistance.

Female AKR/J mice were immunized s.c. with 10^3 viable LVS and 30 days later given an i.p. of 10^6 viable LVS. At 2 to 29 days post-booster, ISC were harvested and groups of syngeneic recipients were injected i.p. with 1×10^8 ISC, alone or concurrently with 10^7 killed SCHU S4 organisms, and treated with streptomycin for 5 days to prevent replication of transferred LVS organisms. Some recipients were challenged i.p. 20 h later with 25 to 50 MLD of strain SCHU S5 and survivors were back-challenged s.c. on day 30 with 100 to 150 MLD of strain SCHU S4. Two other groups of recipients, one of which was treated with streptomycin, were challenged s.c. on day 30 with 100 to 150 MLD SCHU S4. Survival data summarized in Table 3 indicate that:

(i) ISC were able to elicit protection as early as 4 days post-booster; (ii) the ability to express protection was rapidly lost (only 1 of 6 recipients of 6-day ISC survived challenge) and the loss appeared to be correlated with a reduction in number of spleen cell-associated viable organisms; (iii) immune competence of ISC harvested as late as 29 days post-booster could be reactivated by concurrent injection of specific killed antigen; (iv) the high degree of protection afforded by ISC administered concurrently with 10^7 killed F. tularensis was effective for at least 30 days post-transfer; and (v) streptomycin provided effective control of spleen cell-associated LVS organisms. In control groups, one of 26 recipients of NSC and killed antigen and 1 of 36 recipients of killed antigen alone survived primary challenge; all mice in other control groups died within 10 days.

Quantitation of the antigenic requirement for transfer of protection to AKR/J mice by immune spleen cells. AKR/J female mice were vaccinated s.c. with 10^3 LVS and boostered i.p. 30 days later with 10^6 LVS. Nine days later, 1×10^8 ISC were transferred i.p. to syngeneic recipients concurrently with i.p. injection of graded amounts (0 to 10^9) of killed strain SCHU S4 organisms. Comparable groups of recipients were challenged 20 h later by the i.p. or s.c. route with 25 to 50 MLD of strain SCHU S5. All survivors of the 20 h challenge and one group of nonchallenged recipients were inoculated s.c. 30 days later with 100 to 150 MLD of strain SCHU S4. Since previous experiments with LVS-immunized LVS-boostered mice indicated that viable organisms were not detectable in spleen cell suspensions 7 or more days following booster, recipients were not given streptomycin therapy.

No viable spleen cell-associated strain LVS organisms were recovered in cultures from the ISC preparation. All control mice died within 10 days with the exception of four in i.p.-challenge groups, i.e., 1 of 20 inoculated with NSC and killed antigen, 2 of 10 injected with antigen alone and 1 of 30 untreated challenged mice. With one exception (10^2 antigen concentration) survival ratios of the i.p.-challenged recipients represent the combined results of two separate experiments.

Survival ratios (Table 4) indicated that: (i) killed antigen in combination with ISC was required for expression of high grade adoptive immunity; (ii) when the challenge was administered s.c., the maximal effect of antigen was achieved with concentrations of 10^6 to 10^9 killed bacteria but with i.p. challenge while a smaller amount of antigen was required to effect protection, a high concentration (10^9) was possibly suppressive; (iii) protection afforded recipients by ISC in the absence of antigen was adoptive, i.e., survivors were susceptible to backchallenge; and (iv) long lasting high-grade immunity developed in recipients that were given antigen doses $\geq 10^6$ killed bacteria at the time of ISC transfer. Antigen (10^7, 10^8 or 10^9) alone or in combination with NSC conferred essentially no protection against virulent challenge either initially (20 h) or at 30 days post-transfer.

Specificity of the antigenic requirement for transfer of protection to AKR/J recipients of immune spleen cells. In order to determine the immunologic specificity of the antigenic requirement for continued demonstration of adoptive immunity by transferred ISC, the effect of concurrently inoculated killed antigen prepared from F. tularensis SCHU S4 or Y. pestis was compared. Donor AKR/J mice were immunized with 10^3 viable LVS s.c. and boostered one month later with 10^6 viable LVS i.p. Spleen cell

transfer was made 8 days later. Each recipient received approximately 1×10^8 spleen cells i.p. concurrently with an i.p. dose of killed antigen (10^6, 10^7 or 10^3 organisms) contained in 0.2 ml of saline. Recipients were challenged with 25 to 50 MLD of F. tularensis SCHU S5 by the i.p. route on day 1 or 7 following ISC transfer; all survivors and 3 nonchallenged groups were inoculated s.c. approximately one month later with 100 to 150 MLD F. tularensis SCHU S4. Appropriate groups of control mice were included at time of transfer and of challenge; antigen alone or concurrent with NSC had no protective effect.

When challenge was administered by the i.p. route 20 h after spleen cell transfer, Y. pestis antigen over a narrow concentration range was as effective as F. tularensis antigen for potentiating the protective activity of ISC (Table 5). Although at 7 days post-ISC transfer, protection remained enhanced by the 10^7 concentration of heterologous Y. pestis antigen, enhancement was appreciably greater with 10^8 Y. pestis. In contrast to stimulation provided by homologous antigen, the effect of Y. pestis antigen was no longer evident when survivors were backchallenged nor when initial challenge was delayed for 30 days.

In a similar experiment (Table 6), challenge was administered by the s.c. route on days 1, 3, 5, 10, or 14 following spleen cell transfer to syngeneic recipients of ISC, alone or with 10^7 Y. pestis or SCHU S4 killed organisms. In contrast to the high-grade protection potentiated by the homologous F. tularensis antigen, no protective activity by ISC alone or in combination with Y. pestis antigen was observed at any time up to 14 days following cell transfer.

DISCUSSION

The present studies on adoptive transfer of cell-mediated immunity (CMI) clearly demonstrate that recent or continual interaction between an adequate amount of antigen (viable or nonviable) and splenocytes from a syngeneic immune donor is required for transfer of protection against fully virulent F. tularensis. Failure to transfer resistance results if antigenic stimulation of the immune donor is interrupted either because of antibiotic intervention following booster with fully virulent SCHU S4 or because of rapid elimination of attenuated organisms in an LVS booster dose. Similarly, the capacity of ISC to transfer resistance decreases as the interval between donor immunization and splenocyte harvest increases. In each instance, however, donors are resistant to an otherwise fatal infection and their ISC retain the potential to respond to antigen. Immunocompetence of ISC to effect successful transfer of immunity can be demonstrated by concurrent injections of nonprotective ISC and nonviable antigen into recipients that then become resistant to infection.

Present evidence suggests that CMI constitutes the major immune defense against infection with fully virulent F. tularensis (1, 10, 14) or other facultative intracellular microorganisms, i.e., Mycobacterium spp. (2, 5, 30-32, 34), Listeria monocytogenes (8, 16-18, 20-22), Brucella abortus (16) and Salmonella spp. (3, 7). Briefly, according to current concepts (4), the CMI response consists of a sequence of cellular interactions: (i) stimulation of thymus-derived lymphocytes (T-cells), either directly by specific antigen or subsequent to macrophage-processing of antigen, (ii) release

of lymphokines from antigen-activated T-cells, (iii) lymphokine stimulation of cells (macrophages) that lack immunocompetence, and finally (iv) enhanced attachment and killing or inhibition of intracellular organisms by lymphokine-stimulated macrophages.

Active replication and persistence of SCHU S4 organisms in an immunized donor insures prolonged exposure of the sensitized T-cell population to nonprocessed and processed specific antigen, probably resulting in continual lymphokine release and activation of donor macrophages. Persistence of transfer capability for 2 to 3 weeks after viable organisms were no longer detectable suggests that residual antigenic moieties maintain stimulatory activity until fully degraded by the boostered mouse. The effectiveness of the killed antigen treatment of recipients in restoring transfer capability indicates that ISC from resistant donors require exposure to active antigenic moieties, either as residuum or supplement, to demonstrate functional competence.

The quantity of antigen transferred or injected into ISC recipients markedly influences the nature and persistence of the protection conferred. With an optimal amount of antigen supplement, protection of ISC recipients is long lasting and independent of challenge route, whereas with lesser antigenic stimulus it is of brief duration and effective only against i.p. challenge. Localized macrophage activity resulting from lymphokine release by antigen-stimulated immune T-cells may account for short-term resistance, but the mechanism for a long-lasting antigen effect is unclear. Nonviable antigen alone, or administered concurrently with NSC, cannot induce active immunity. Residual or supplemental antigen administered with ISC, however,

may exert its effect by inducing proliferation of immunocompetent donor T-cells (12), by prolonging their functional survival (11) and/or by stimulating production and release of relatively stable intermediate products (13, 15, 16, 19, 23).

Factors contributing to enhancement of protection against tularemia by treatment of ISC recipients with killed Y. pestis are as yet undefined. Evidence does not support the hypothesis that a specific response due to antigenic cross-relationships is involved. There is no known serological cross-reaction between Y. pestis and F. tularensis and an optimal stimulating dose of Y. pestis (10^7 organisms) fails to protect against s.c. challenge with F. tularensis or to act as a primer to ISC for induction of persistent immunity in recipients. Moreover, stimulation of resistance to F. tularensis, identical in all respects to that reported with Y. pestis, can be demonstrated with a comparable number of killed Salmonella typhi or Bordetella pertussis organisms (unpublished data), further suggesting the involvement of non-specific factors.

Enhancement of protection by heterologous bacteria occurred only if all reactants, i.e., transferred ISC, killed Y. pestis organisms and virulent SCHU S5 challenge organisms, were administered to the recipient by the i.p. route, and if at least 20 h intervened between treatment with ISC and killed bacteria and challenge with F. tularensis (unpublished data). These requirements and the nonspecific nature of the stimulus suggest a major role for the peritoneal macrophage population that probably consists of both donor and resident macrophages. The effector cell may well be of donor origin.

Other investigators using in vitro techniques have reported that murine peritoneal macrophages from immunized donors can inhibit and at times destroy phagocytized virulent F. tularensis organisms, whereas macrophages from nonimmune donors are unable to prevent proliferation of the ingested bacteria (33). Nonimmune macrophages appeared to have little effect on proliferation of the virulent challenge because recipients pretreated with killed Y. pestis alone or in combination with NSC reacted in the same manner as untreated mice. In ISC recipients the killed nonspecific antigen may be phagocytized more readily by macrophages that were activated earlier in the boostered donor. This additional stimulus could increase the level of metabolic activity in donor macrophages (24) or contribute to their retention in the peritoneal activity where they would be available to phagocytize the subsequently administered virulent organisms and prevent dissemination to other host tissues. Processed antigen from virulent organisms killed during their intracellular residence could provide specific stimulation to donor ISC and initiate lymphokine release for activation of additional macrophages. The newly-activated macrophages would be available for processing organisms that proliferated in, and were released from, resident macrophages or survived intracellular residence in "immune" macrophages. Studies are currently in progress to examine more closely the sequence of events that follows i.p. transfer of lymphoid cells from immune donors and i.p. treatment of recipients with nonviable homologous or heterologous antigen.

The present data indicating that small numbers of viable cell-associated organisms in transferred ISC markedly influence resistance of recipients against F. tularensis suggest that CMI in infections with other facultative

intracellular bacteria likewise may be dependent on continuous antigenic stimulation of immune lymphocytes. In studies on the role of CMI in listeriosis (8, 16, 18, 20-22), brucellosis (17), tuberculosis (2, 5, 18, 20, 30-32) and salmonellosis (3, 6, 7), relatively large numbers of viable organisms were present in the transferred ISC and/or in the challenge dose. While in vitro exposure of ISC to specific antibiotic could effect significant reduction in the number of cell-associated Listeria, 40 to 240 viable organisms remained for transfer with the ISC (35); these replicating organisms supplemented by antigen from antibiotic-inactivated bacteria probably supplied the stimulus for the transferred immune cells. The murine tularemia model is the only system presently available in which investigations on adoptive transfer of resistance to infection can be performed without the complicating effects of large numbers of cell-associated replicating organisms.

TABLE 1. Influence of streptomycin treatment of immune donor mice on the number of viable spleen cell-associated SCHU S4 organisms transferred to recipients and on survival of recipients after challenge with 25 MLD of streptomycin-resistant SCHU S5

Streptomycin therapy schedule (days)[a]		Live SCHU S4 in donor spleen cells transferred on day 12	Recipient survivors/total	
Donor	Recipient		No Challenge	SCHU S5 Challenge
3-5	None	0	6/6	1/6
6-8	None	6	6/6	0/6
6-11	None	3	6/6	1/6
6-11	0-4	3	5/6	0/6
9-11	None	14	3/6	1/6
None	None	500	0/6	0/6
None	0-4	500	--	4/6

[a] Therapy schedules: donors, days post-booster; recipients, days after spleen cell transfer. Mice were administered 400 µg of streptomycin (0.1 ml s.c.) every 8 h on days indicated.

TABLE 2. Number of cell-associated viable SCHU S4 organisms in splenocyte harvests 4 to 47 days after booster inoculation with F. tularensis SCHU S4 and ability of splenocytes to transfer protection to syngeneic recipients[a]

Post booster (day)	No. donor cells transferred		Survivors/ total
	Spleen cells (x 10^7)	Spleen cell associated live F. tularensis	
4	20	10^4	10/10
6	6	10^6	0/10
8	10	10^2	10/10
10	14	50	8/10
12	7	16	9/10
14	11	86	7/10
18	10	0	10/10
21	8	1	6/10
35	7	0	4/10
45	6	0	1/16
47	7	0	0/7
47	7 + 10^7 Ag[b]	0	7/8

[a] AKR/J recipients administered 400 µg streptomycin twice daily for 5 days post-transfer and challenged i.p. with 30 MLD of streptomycin-resistant F. tularensis SCHU S5 at 20 h post-transfer.

[b] 10^7 washed phenol killed SCHU S4 organisms administered i.p. at time of spleen cell transfer.

TABLE 3. Effect of killed Francisella tularensis antigen[a] on protection of AKR/J recipients of syngeneic spleen cells from LVS vaccinated-LVS boostered donors.

Cell transfer post-booster (day)	No. donor cells transferred		Survivors/total of recipients after virulent F. tularensis challenge							
			SCHU S5 i.p. 20 h post transfer				SCHU S4 s.c. 30 days post transfer			
			Initial challenge[b]		SCHU S4 back-challenge of survivors		Recipient treatment			
							No antibiotic		Streptomycin[b]	
	Spleen cells (x 10^7)	Spleen cell-associated live LVS	No antigen	10^7 antigen	No antigen	10^7 antigen	No antigen	10^7 antigen	No antigen	10^7 antigen
2	12.0	13	1/6	0/6	0/1	NA	4/5[c]	4/4[c]	0/6	5/6
4	9.2	9	8/10	10/10	0/8	10/10	3/6	6/6	0/6	5/6
6	7.2	0	1/6	5/6	0/1	5/5	1/6	5/6	0/6	6/6
8	8.4	0	2/5	4/6	0/2	4/4	0/6	6/6	0/6	6/6
9	8.2	0	0/10	10/10	NA	9/10	0/6	6/6	ND	ND
10	6.2	0	0/10	9/10	NA	9/9	0/6	4/6	ND	ND
29	6.2	0	1/8	7/8	ND	ND	ND	ND	ND	ND

[a] 10^7 washed phenol killed SCHU S4 antigen injected i.p. at time of immune spleen cell transfer.

[b] AKR/J recipients administered 400 µg of streptomycin s.c. every 12 h for 5 days post-transfer.

[c] Three of 12 recipients of 2-day post-booster spleen cells died within 10 days of transfer.

TABLE 4. Quantitation of antigenic requirement for effecting adoptive transfer of protection to AKR/J recipients of syngeneic spleen cells from LVS immunized donors[a]

Antigen dose (killed SCHU S4 organisms)	Survivors/total after challenge				
	Intraperitoneal		Subcutaneous		
	20 h challenge[b]		20 h challenge[b]		30-day primary[c] challenge
	Primary challenge	Backchallenge of survivors[c]	Primary challenge	Backchallenge of survivors[c]	
None	8/20	0/8	0/10	--	0/10
10^2	7/10	0/7	0/10	--	0/10
10^4	10/20	1/10	2/10	2/2	3/10
10^6	16/20	14/16	7/10	7/7	6/10
10^7	18/20	18/18	9/10	9/9	4/10
10^8	14/20	13/14	8/10	8/8	9/10
10^9	5/20	5/5	6/10	6/6	8/10

[a] Donors vaccinated with 10^3 LVS s.c., and boostered 30 days later with 10^6 LVS i.p.; 10^8 spleen cells transferred 9 days post-booster.

[b] Recipients challenged with 25 to 50 SCHU S5 organisms.

[c] Challenged s.c. with 100 to 150 SCHU S4 organisms.

TABLE 5. Antigenic enhancement of adoptively transferred immunity against i.p. challenge with virulent *F. tularensis* in AKR/J recipients of syngeneic immune spleen cells (ISC)

Recipient treatment[a] ISC	Antigen dose	Survivors/total for groups challenged post-spleen cell transfer by days				
		Day 1		Day 7		Day 30
		Initial challenge	30-day backchallenge	Initial challenge	30-day backchallenge	Initial challenge
Alone	None	3/10	0/3	1/10	1/1	0/9
+ *F. tularensis* antigen	10^7	8/10	8/8	10/10	10/10	8/10
+ *Y. pestis* antigen	10^6	4/10	1/4	1/10	0/1	--
	10^7	8/10	0/8	6/10	0/6	0/9
	10^8	3/10	1/3	9/10	0/9	--

[a] 10^8 immune spleen cells transferred i.p. 8 days post-LVS booster; *Y. pestis* or *F. tularensis* killed antigens administered i.p. at time of cell transfer. All recipients of normal spleen cells, with or without antigen, or of antigen alone died within 8 days after challenge.

TABLE 6. Specificity of antigenic requirement for adoptive transfer of immunity against s.c. challenge

Day of SCHU S5 challenge after ISC transfer	Survivors/total for ISC-recipient groups[a]			
	Killed antigen concomitant with ISC			
	None	10^7 *Y. pestis* organisms	10^7 *F. tularensis* organisms	
			Primary challenge	30-day backchallenge
1	0/10	0/10	6/10	6/6
3	0/10	0/10	8/10	8/8
5	0/10	0/10	10/10	10/10
10	0/10	0/10	10/10	9/10
14	0/10	0/10	9/10	9/9

[a] 10^8 spleen cells transferred i.p. 8 days after LVS booster; *Y. pestis* or *F. tularensis* antigens administered i.p. at time of cell transfer. All recipients of normal spleen cells, with or without antigen, or of antigen alone died within 8 days post challenge.

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