IMMUNITY AGAINST TULAREMIA: PASSIVE PROTECTION OF MICE BY TRANSFER OF IMMUNE TISSUES

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Two types of immune response may lead to a state of acquired resistance to tularemia in the mouse. The first type develops only after infection with strains capable of proliferation *in vivo*, allows survival of the host after challenge with strains of full virulence, and cannot be passively transferred by means of serum. The second type develops after immunization with non-viable antigens as well as after infection, protects only against strains of less than full virulence, and is readily transferred by means of serum. The available evidence suggests that similar considerations apply with respect to acquired immunity against tularemia in guinea pigs, rabbits, monkeys, and probably man. The rat responds differently; this more resistant animal may survive challenge with fully virulent strains after active immunization with non-viable vaccines, or after passive transfer of immune serum.

Immunity of the second type is accounted for adequately by the action of humoral antibody directed against the somatic polysaccharide of the organism (1, 2). The first type of immunity, however, does not appear to be associated with serum antibody, and presumably is dependent upon a state of altered reactivity of the tissues. Similar considerations apply to immunity in brucellosis and tuberculosis (3–5) and it has been demonstrated that acquired resistance to the latter disease may be transferred by means of phagocytic cells (6). The present investigation was undertaken to test the hypothesis that the immunity that is effective against fully virulent strains of *Pasteurella tularensis* may be transferred by cells.

Materials and Methods

Mice.—Inbred A/LN strain mice procured from the National Institutes of Health, Bethesda, and a Fort Detrick strain (FD) of non-inbred Swiss mice were used. The A/LN mice were segregated as to sex and age, and only females were used as donors or recipients of transferred tissues. For experiments not involving tissue transfer, males or females were used indiscriminately.

Bacterial Cultures.—A parent Jap strain of *P. tularensis* was used for immunization of mice. In our hands, this strain had an LD_{50} for normal mice of approximately 10^4 organisms by the intraperitoneal route and 10^6 organisms by the subcutaneous route. The strain was grown and maintained on slants of SB agar (7).

Two strains of *P. tularensis* were used for challenge. The Schu S4 strain, susceptible to

streptomycin, and the Schu S5 strain, resistant to streptomycin, were highly virulent for mice, guinea pigs, and rabbits. 1 organism constituted 1 LD_{50} for mice for both of these strains by either subcutaneous or intraperitoneal injection. The challenge strains were maintained in a lyophilized state. Working stock suspensions were grown first on SB agar and then in a modified casein hydrolysate medium similar to that described by Mills *et al.* (8). Fresh stock suspensions were prepared every 4 to 5 weeks and held at 4 to 6°C. Dilutions for challenge were made in sterile gelatin saline, containing 0.9 per cent sodium chloride and 0.1 per cent gelatin. Virulence of these challenge strains was checked periodically in rabbits. They were considered fully virulent if more than 50 per cent of the rabbits died of tularemia within 10 days following subcutaneous injection of 5 to 10 organisms.

The EV-76 strain of *Pasteurella pestis*, grown on slants of blood agar base (Difco) at 21°C for 24 hours, was maintained on this medium at 4 to 6°C.

Immunization of Donor Mice.—Mice 8 weeks or more old were immunized by infection with *P. tularensis*, strain Jap. Primary immunization consisted of approximately 5×10^3 organisms injected by the intraperitoneal route. A booster dose of approximately 5×10^7 organisms was injected by the intraperitoneal route 4 to 5 weeks after the primary dose. These mice are hereafter referred to as immune mice and their tissues as immune tissues.

Mice were immunized with a killed tularemia vaccine by intraperitoneal injection on 5 successive days of 0.5 ml doses of a suspension of acetone-killed *P. tularensis* strain Schu S5, at a concentration of 4×10^{10} cells per ml. 14 days after the initial series the mice were similarly injected with a single booster dose of this vaccine.

Mice were immunized with *P. pestis* strain EV-76 by a subcutaneous injection of approximately 1×10^7 viable organisms and 28 days later received 1×10^8 viable organisms by the intraperitoneal route.

Harvest and Transfer of Tissues.—Peritoneal leucocytes were obtained from immunized or normal donor mice 4 to 6 days after an intraperitoneal injection of 1 ml of 0.9 per cent saline. Immunized donors received a booster injection of vaccine 2 to 4 days before the saline injection. The mice were anesthetized by subcutaneous injections of sodium pentobarbital, exsanguinated by cardiac puncture, and the peritoneal cavities injected with 8 ml of Hanks's balanced salt solution (BSS). The abdomens were kneaded gently and the suspended cells were withdrawn by a syringe and needle. Leucocytes were counted, centrifuged at 500 *g* for 5 minutes, and resuspended in fresh, cold BSS to the desired concentration. Recipients were injected intraperitoneally with 1 ml of the leucocyte suspension within 6 hours after harvest.

For the passive transfer of spleen tissue or other reticuloendothelial (RES) tissue, donor mice were anesthetized, exsanguinated, and the organs excised. The organs were immediately washed in cold BSS, placed in shallow dishes containing BSS plus 10 per cent horse or bovine serum, and minced and teased apart with surgical scalpels. Crushing of tissues was carefully avoided. Large pieces of debris were removed by filtering the suspension through a 200 mesh stainless steel wire sieve. The resulting filtrate contained individually suspended cells and aggregates of up to 20 cells per clump. Total cells and leucocytes were counted, centrifuged at 500 *g* for 5 to 10 minutes, and resuspended in cold BSS to the desired concentration. Recipient mice, 6 to 8 weeks old, were injected intraperitoneally with 1 ml of the tissue suspension.

Viability of the tissues was determined at the time of transfer by suspending cells in 0.9 per cent saline containing 1 per cent trypan blue and immediately counting the percentage of colorless, and hence viable, cells.

Measurement of Resistance to Tularemia.—Mice were challenged intraperitoneally or subcutaneously with *P. tularensis* and observed for deaths daily for 28 days. Death times and survivors were appropriately combined into a single value, the mean reciprocal death time (MRDT), in a manner similar to that used by Brownlee and Hamre (9). The MRDT and

standard deviation were calculated for each challenged group. For example, death times of 6, 7, 8, 8, 8, 8, 9, 9 days and 2 survivors for a group of 10 mice would have an MRDT equal to $\frac{1/6 + 1/7 + 4/8 + 3/9 + 2/i}{10}$, or 0.103. The longer the animals survived, the smaller was the MRDT. It was assumed from previous experience that mice surviving 28 days after challenge would have a death time of infinity for these experiments. Differences in resistance between 2 groups of animals, as measured by MRDT's, were tested for significance by the t test (10).

Hemagglutination Titers.—These were determined according to the procedure of Wright and Feinberg (11), using sheep erythrocytes sensitized with *P. tularensis* polysaccharide as the test antigen. The highest dilution of serum producing a uniformly dispersed pattern of erythrocytes was recorded as the antibody titer.

EXPERIMENTAL

Transfer of Peritoneal Leucocytes.—Leucocytes from the peritoneal cavities of normal and immune FD mice were suspended in 2 parts BSS and 1 part normal or immune serum and injected intraperitoneally into groups of normal FD mice. 24 hours later these animals plus recipients of immune serum were challenged by the intraperitoneal route with approximately 135 LD_{50} of *P. tularensis* strain Schu S4. Groups of untreated mice and immune mice were challenged similarly.

Examination of stained preparations of the peritoneal leucocytes revealed lymphocytes and monocytes in approximately equal numbers, and less than 1 per cent of other leucocyte types. No significant differences in cell type or in cellular morphology were observed between leucocytes from normal and from immune donors.

The ratios of deaths to total numbers of mice challenged are presented in Table I along with mean reciprocal death times (MRDT) and their standard deviations (S.D.). Using the t statistic and a probability of error of 0.05, it was determined that recipients of immune peritoneal leucocytes were significantly more resistant to tularemia than all other groups of recipient mice and untreated mice, but were obviously less resistant than immune donor mice. Recipients of 0.33 ml of immune serum only were not significantly more resistant than recipients of normal serum and normal cells, but were significantly more resistant than previously untreated mice. In additional experiments this slight resistance of animals inoculated with immune serum was not increased appreciably when the volume of antiserum was increased to 1.0 ml. No animal that received immune serum alone survived challenge.

Three of 30 recipients of immune leucocytes survived challenge, and these animals were rechallenged in the same manner after 6 weeks. All animals died at the same rate as the normal mice, indicating that infection had been absent or minimal following the initial challenge, since no active immunity developed. The initial passive immunity was evidently of relatively short duration in these non-inbred mice. Previous experience had indicated that active immunity

in mice persisted at least 3 months after recovery from infection with the Jap strain of *P. tularensis*.

Consideration was given to the possibility that the enhanced resistance of recipient mice resulted from infection with organisms present in the leucocyte suspension. However, no colonies of *P. tularensis* were cultivated by incubation of the immune leucocyte suspension on SB agar plates, and normal mice failed to show a significant resistance to tularemia 24 hours after intraperitoneal injection of viable Jap strain organisms. For these reasons the observed resistance was believed to be caused by factors other than an active immunity.

TABLE I

Passive Transfer of Peritoneal Leucocytes

Recipients received:		Dead/Tested	MRDT (±S.D.)§
Cells*	Serum‡		
Immune	Immune	14/15	0.108 (±0.039)
Immune	Normal	13/15	0.141 ±0.061)
None	Immune	15/15	{ 0.191 (±0.015) }
Normal	Immune	14/14	{ 0.192 (±0.023) }
Normal	Normal	12/12	{{0.215 (±0.026)}}
None	None	16/16	{{0.235 (±0.024)}}
Immune donor mice		0/15	0

* 3.4×10^7 peritoneal leucocytes from immune or normal mice respectively.

‡ 0.33 ml of serum from indicated donor transferred. Hemagglutination titer of immune donor serum was 1:1280.

§ Mean of reciprocals of death times and standard deviation. All mice were challenged 24 hours after tissues were transferred with 135 LD_{50} of *Pasteurella tularensis* strain Schu S4 by the intraperitoneal route. Data enclosed by brackets are not significantly different at $P = 0.05$.

Transfer of Cells from Spleens and Other Organs.—When it was shown that transfer of immune peritoneal leucocytes could enhance the resistance of recipients, other tissues of the reticuloendothelial system (RES) of immune mice were investigated in a similar manner. These tissues included spleen, liver, mesenteric lymph nodes, lung, and sternal bone marrow. Resistance to tularemia was increased among recipients of immune spleen tissue and to a lesser degree among recipients of lymph node tissue. No increase in resistance was observed among recipients of other transferred tissues. The passively acquired resistance of recipients of immune spleen cells equalled and often exceeded the previously described resistance of recipients of immune peritoneal leucocytes. High concentrations of cells were readily obtained from spleen tissue, and accordingly spleen cells were used in subsequent experiments.

Spleens removed from immune donors between 5 and 12 days after a booster dose of live vaccine were usually 2 to 3 times the size of normal spleens. Thereafter the size of the spleen returned to normal. The ability of suspensions of spleen tissue to transfer resistance to tularemia appeared to be maximal during this interval of splenomegaly.

TABLE II

Resistance of Recipients of Passively Transferred Viable Spleen Tissue and Serum to Subcutaneous and Intraperitoneal Challenge

Number of spleen cells transferred	Serum transferred	Route of challenge*	Dead/ Tested	MRDT (±S.D.)‡
		Recipients of immune spleen cells		
1.8×10^8	Immune§	Intraperitoneal	6/10	0.070 (±0.064)
1.8×10^8	Normal	Intraperitoneal	8/10	0.093 (±0.051)
0	Immune	Intraperitoneal	10/10	0.187 (±0.017)
1.8×10^8	Immune	Subcutaneous	10/10	0.111 (±0.009)
1.8×10^8	Normal	Subcutaneous	10/10	0.124 (±0.015)
0	Immune	Subcutaneous	10/10	0.131 (±0.059)
		Recipients of normal spleen cells		
8.4×10^7	Immune	Intraperitoneal	10/10	0.192 (±0.021)
8.4×10^7	Normal	Intraperitoneal	15/15	0.233 (±0.028)
9.2×10^7	Immune	Subcutaneous	8/8	0.118 (±0.007)
9.2×10^7	Normal	Subcutaneous	8/8	0.168 (±0.015)
Untreated		Intraperitoneal	10/10	0.245 (±0.015)
Untreated		Subcutaneous	8/8	0.171 (±0.009)

* Mice challenged with 72 LD_{50} of *Pasteurella tularensis*, strain Schu S5, 3 days after passive transfer of tissues or serum.

‡ Mean of reciprocals of death times and standard deviation. Data enclosed by brackets were not significantly different, $P = 0.05$.

§ 0.25 ml serum transferred. Immune homologous mouse serum had a hemagglutination titer of 1:2560.

In evaluating the resistance observed in recipient mice consideration was given again to the possibility of active immunity developing as a result of simultaneous transfer of intracellular, viable *P. tularensis*. This possibility was apparently eliminated by suspending the spleen cells in BSS containing 100 μg of dihydrostreptomycin and 10 units of penicillin per ml, the latter included to control other contaminants. After the cells were treated with antibiotics and washed, no viable *P. tularensis* was recovered by plating, although viable organisms were usually present initially. As a further precaution against infection, recipients were treated with 400 μg of streptomycin per day by subcutaneous injection until 24 hours before challenge, but for no longer than

12 days following transfer of tissues. All other challenge groups were similarly treated unless otherwise stated. The streptomycin-resistant Schu S5 strain was used for challenge to avoid possible effects of residual streptomycin.

The experimental data presented in Table II are typical of results obtained after challenge of recipients of passively transferred spleen tissue. No significant difference was observed between the resistances of recipients of normal spleen cells with normal serum and untreated mice. By subcutaneous challenge, no significant difference was observed between the resistance of recipients of immune spleen cells and recipients of immune sera, although a decided difference in resistance between these groups was observed if challenge was by the intraperitoneal route. These results were confirmed in replicate experiments.

TABLE III

Passively Transferred Graded Doses of Immune Spleen Cells and Killed Immune Spleen Cells

Number of spleen cells transferred	Dead/Tested	MRDT (±S.D.)*
Recipients of viable immune spleen cells		
1.1×10^8	6/8	0.095 (±0.065)
1.1×10^7	10/10	0.153 (±0.015)
1.1×10^6	10/10	0.215 (±0.024)
0	10/10	0.245 (±0.015)
Recipients of killed immune spleen cells‡		
1.1×10^8	9/9	0.250 (0)

* Mean of reciprocals of death times and standard deviation. Mice were challenged with 150 LD_{50} of *Pasteurella tularensis*, strain Schu S5, by the intraperitoneal route 7 days after passive transfer of tissues.

‡ Cells killed by heating at 56°C for 30 minutes.

Effect of Graded Doses of Immune Spleen Cells.—The degree of resistance resulting in recipient A/LN mice after the passive transfer of graded doses of spleen cells from immune isologous donors is presented in Table III. Resistance appeared to be directly proportional to the $\log_{10}$ number of viable, immune spleen cells transferred. Extrapolation of these data suggests that transfer of 1 billion or more spleen cells would allow essentially complete survival of recipients after intraperitoneal challenge. Transfer of such large numbers of cells was not technically feasible. In order to protect a small percentage of recipients completely and consistently against challenge, however, 10^8 or more spleen cells from immune donors were required, and in several experiments the survivors in such groups ranged from 10 to 70 per cent.

Data presented in Table III also indicate that transfer of killed immune spleen cells did not increase the resistance of recipients. In other experiments immune spleen cells that were killed by crushing or by repeated freezing and

thawing were also inactive. It would appear that the immunity transmitted by phagocytic cells was associated with only viable tissues, and hence is not a result of humoral antibody.

The duration of passively acquired resistance to tularemia was investigated in inbred, A/LN strain recipients by challenging 1, 3, 7, and 21 days following the passive transfer of 1.1×10^8 immune spleen cells. Homologous Fort Detrick strain recipients were compared with isologous A/LN strain recipients at 2 of the 4 intervals. Untreated mice of these 2 strains were previously determined to be equally susceptible to similar doses of *P. tularensis* administered subcutaneously or intraperitoneally. The results presented in Table IV indicated that the per cent survival among groups of A/LN recipients was approximately

TABLE IV

Resistance to Tularemia at Intervals Following Passive Transfer of Spleen Cells from Immune A/LN Donor Mice to Normal A/LN and Fort Detrick Recipient Mice

Recipients	Intervals between transfer and challenge							
	1 day		3 days		7 days		21 days	
	D/T*	MRDT‡	D/T	MRDT	D/T	MRDT	D/T	MRDT
A/LN (inbred)	6/8	0.084	7/10	0.068	6/8	0.094	9/13	0.103
F.D. (non-inbred)			6/6	0.129			7/7	0.214
Untreated (A/LN)	10/10	0.245	10/10	0.240	10/10	0.245	10/10	0.240

* Ratio of deaths to total number challenged.

‡ Mean of reciprocals of death times. Mice challenged with 50 to 150 LD_{50} doses of *Pasteurella tularensis* strain Schu S5, by the intraperitoneal route.

the same at the 4 intervals tested. All of the Fort Detrick strain of recipients succumbed to tularemia after challenges at 3 and 21 days post-transfer. Using the MRDT as a measure of resistance, it appeared that by 21 days post-transfer, the non-inbred recipients lost nearly all of the resistance demonstrated at 3 days post-transfer. No such sharp decrease was noted in the resistance of inbred recipients over the same time period. This difference in resistance between isologous and homologous recipients suggests that a tissue transplantation immunity or homograft reaction developed in non-inbred recipients. Such a phenomenon could cause early rejection of transferred viable tissues from genotypically heterologous mice (12) and thereby reduce the efficiency of the resistance mechanism associated with the transferred cells. Proof of this must await further experimentation.

Transfer of Tissues from Donors Immunized with Other Vaccines.—The transfer of isologous spleen cells from mice immunized with either non-viable *P. tularensis* or with viable *P. pestis* vaccines served as controls for the immuniza-

tion techniques used in the previous experiments. Donors, recipients, and previously untreated mice were challenged intraperitoneally with the Schu S5 strain on the 3rd day post-transfer. Resistance was no greater among mice immunized with viable *P. pestis* and recipients of spleen tissue and/or serum from these animals than it was among untreated mice and, hence, need not be described here in detail. As expected, all mice succumbed to tularemia following challenge. Only those mice immunized with the non-viable *P. tularensis* vaccine and recipients of serum from donors similarly immunized died significantly later than previously untreated controls (Table V). These results suggest that passive transfer of cellular immunity to tularemia was possible only if donors were specifically immunized by infection with *P. tularensis*.

TABLE V

Passive Transfer of Tissues and Serum from Donors Immunized with a Killed, Whole-Cel Pasteurella tularensis Antigen

Passively transferred:	Dead/Tested	MRDT (±S.D.)*
1.6×10^8 spleen cells	10/10	0.225 (±0.026)
1.3×10^7 peritoneal leucocytes	10/10	0.235 (±0.005)
Donor serum (0.25 ml)‡	10/10	0.188 (±0.021)§
Donor mice	8/8	0.161 (±0.011)§
Untreated	10/10	0.240 (±0.021)

* Mean of reciprocals of death times and standard deviation. Mice challenged with 82 LD_{50} doses of *Pasteurella tularensis* by the intraperitonal route 3 days after transfer of tissue.

‡ Hemagglutination titer of donor serum was 1:640.

§ Significantly different from untreated mice, $P = 0.05$.

DISCUSSION

The data indicate clearly that immunity to fully virulent strains of *P. tularensis* can in fact be transferred to normal animals by tissues from an immune donor. The following results appear to eliminate the possibility that this immunity was a result of active immunization by the simultaneous transfer of viable *P. tularensis* or its antigens: (*a*) no viable *P. tularensis* were recovered from antibiotic-treated donor cells, (*b*) resistance was transferred with viable spleen cells but not with cells inactivated by freezing and thawing, a procedure that is not especially detrimental to *P. tularensis*, and (*c*) resistance did not persist in non-inbred recipients as long as in isologous recipients.

A significant percentage of recipients of immune cells survived intraperitoneal challenge and as a group were considerably more resistant than recipients of immune sera. Following subcutaneous challenge, however, all recipients of immune cells died and were no more resistant than similarly challenged recip-

ients of immune sera. These results might be explained by postulating that the transferred immune cells tended to remain in the peritoneal cavity or its efferent lymphatics. Under these conditions the immune cells would be effective against intraperitoneally injected organisms, but not against challenge at a remote site such as the skin.

In all cases in which a considerable degree of immunity was transferred by means of spleen cells, splenomegaly was observed in donor mice. Because this splenic hyperplasia was evident in the gross only in mice immunized with infectious *P. tularensis* it was inferred that, in part, this condition represented the host's response to tularemia and was possibly associated with the proliferation of immune cells. As the splenomegaly subsided, these cells may have disseminated throughout the host, thereby reducing the ratio of immune to normal cells in this organ. The resistance of immune donor mice was invariably greater than that of recipients of their spleen cells. A certain loss of efficiency would be expected for cells that had been removed by mincing an intact organ such as the spleen and dispersed into an alien environment such as the peritoneal cavity of another host. This disadvantage appeared to be partially overcome by transferring large numbers of immune cells. In fact, the resistance of the recipients was proportional to the number of immune cells transferred.

Other reticuloendothelial tissue of immune mice besides spleen and peritoneal leucocytes might also contain immune cells but not in sufficient numbers to be detectable by the technique used here. It would be unnecessary, however, to postulate that all tissues of mice become immune following a tularemia infection. The action of serum antibody would tend to localize the infectious agent in the sinuses of RES organs where they would be destroyed or rendered innocuous by fixed or migrating immune phagocytic cells.

Tularemia in mice has definite advantages as a model for study of cellular immunity. Brucellosis and tuberculosis, frequently used in such studies, are chronic or sublethal in most hosts unless infection is initiated with massive numbers of organisms. In the case of tularemia, however, rapidly progressive and uniformly fatal infections may be initiated in normal mice with small numbers of organisms. Antibiotic-resistant and antibiotic-susceptible strains of *P. tularensis* of graded virulence are available, and laboratory personnel may be safely and effectively immunized through the use of a recently developed viable vaccine (13, 14).

SUMMARY

Acquired resistance to fully virulent strains of *P. tularensis* was passively transferred to normal mice by viable spleen cells or peritoneal leucocytes from donors that recovered from infection with an attenuated strain of *P. tularensis*. This passively transferred resistance was reflected in survival or delayed death after challenge of the recipients. The degree of passively transferred resistance

was dependent upon the immune status of the donors and the number of viable immune cells transferred. There were indications that this resistance persisted only as long as the transferred tissues were compatible with the tissues of the recipient. The results support the hypothesis that immunity of mice against fully virulent strains of *P. tularensis* is associated with an altered state of the tissues.

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