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Mechanisms of long and short term immunity to plague

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Summary. Long and short term immunity to plague was produced in normal mice by using, respectively, an antibiotic resistant *Yersinia pestis* and *Yersinia pseudotuberculosis*. Both immunogens were used live. Passive serum transfer experiments, together with assays for the bactericidal activity of macrophages and delayed hypersensitivity tests, showed that the short term immunity was of a humoral nature and the long term immunity was cell mediated. The plague virulence markers of the two immunogens were: *Y. pestis* VW− F1+ P1+ P+; *Y. pseudotuberculosis* VW+ F1− P1− P−. The challenge organism was *Y. pestis* VW+ F1+ P1+ P+.

INTRODUCTION

Since the extensive studies of Meyer and his associates (Meyer, 1953), it has been considered that the main mechanism of immunity to plague is due to humoral antibodies to the virulence factor Fraction I (F1) although V antigen (Lawton, Erdman & Surgalla, 1963) and another uncharacterized immunogen (Thal, 1955) have also been implicated. The antigen reported by Thal (1955), is shared by *Yersinia pestis* and VW− F1− *Y. pseudotuberculosis*, and stimulates long term protection against plague, which suggests the involvement of this antigen in the stimulation of cell-mediated immunity (CMI).

Our present studies using a VW− F1+ P1+ P+ *Y. pestis* and a VW+ F1− P1− P− *Y. pseudotuberculosis* as immunogens, suggest a possible co-operation between humoral and CMI mechanisms against plague.

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MATERIALS AND METHODS

Mice

Four-week-old normal mice of a random stock (ddY/S) of either sex were used.

Bacteria

Yersinia pestis strain Yreka VW+ F1+ P1+ P+, kindly supplied by the late K. F. Meyer was used as the challenge organism. This strain consistently gave a LD_{50} of less than 10 organisms, when injected subcutaneously (s.c.) into mice. A VW− F1+ P1+ P+ mutant strain SCY was obtained, when we introduced a streptomycin (SM), chloramphenicol (CM) and tetracyclin (TC) resistant R-plasmid NR1 from *Escherichia coli* K12 $met^-thr^-leu^-VB_1^-$ auxotroph 921 strain into Yreka by conjugation and selection on SM (50 μg/ml) CM (25 μg/ml) containing threonine and leucine deficient synthetic agar medium. A VW+ F1− P1− P− *Y. pseudotuberculosis* (serovar II) strain 222/+

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was kindly supplied by T. W. Burrows of the Microbiological Research Establishment, Porton, Wilts., England.

Y. pestis virulence determinants VW, P1 and P were reassayed by us on every bacterial strain used, according to the method of Surgalla, Beeseley & Albizo (1970). The envelope antigen F1 was detected by slide agglutination of bacteria grown at 37° with monospecific serum, according to the method of Wake, Misawa & Matsui (1975). A lyophilized stock of each strain was recovered on brain heart infusion agar (BHIA, Difco) plates several days before use, and only typical colonies were further cultured on trypticase soy agar (TSA, BBL) slants at 27°.

When it was necessary to count the number of bacteria inoculated, 0·1 ml of each appropriately diluted bacterial suspension was spread over 5 BHIA plates, and incubated at 27° for 3 days. The average number present was calculated from the number of colonies found (CFU).

Immunization of mice

A 2 day slant culture of SCY grown at 27° was suspended in sterile saline and used to immunize mice intravenously (i.v.) with about 10^5 CFU. Similarly grown cultures of 222/+ were suspended in the iron chondroitin sulphate preparation, Blutal (Blutal, Dainippon Seiyaku, Osaka, Japan) to give about 10^3 CFU/ml. For the immunization of mice with this strain 0·1 ml was injected subcutaneously. Preliminary tests showed that these immunization procedures produced practically no deaths, and gave the best protection (80–100% survivals) against a 100 LD_{50} s.c. challenge dose of Yreka after 2 weeks.

Passive immunization test

Immunized mice were bled from the femoral artery 2 weeks after immunization and the separated serum was pooled. Half ml of each serum was injected i.v. 1 h before challenge with Yreka. For the comparison with the immunity produced in convalescent animals, mice which survived the preceding active immunization followed by Yreka challenge, were bled, and 0·25 ml of the separated serum was injected i.v. into each mouse of one of the control groups in this experiment.

Measurement of bactericidal activity of macrophages (spleen cell fraction bactericidal test, SCFBT)

In the first stage of preliminary tests for the development of this assay method, an SM-resistant *Y. pestis* mutant selected from Yreka on SM-containing agar was used as an indicator strain. This was replaced by SCY after its isolation.

The indicator strains suspended in 0·1 ml saline containing SM (or SM and CM) were injected i.v. into mice. At predetermined intervals, 10 mice were killed with ether and their spleens removed. The spleens were cut with scissors and squeezed gently with sterile forceps in centrifuge tubes containing antibiotic (0·5 mg of SM) and anticoagulant (1·5 mg of potassium oxalate and 1·25 mg of ammonium oxalate) in 10 ml saline to release and suspend the cells. Practically all the spleen cells were released in this way. After washing the cells and centrifugation at 800 *g* for 6 min, the collected spleen cells were resuspended in 4 ml BHIA containing SM (or SM and CM) melted by heating at 50°. These suspensions were quickly overlayed on to BHIA plates containing SM (or SM and CM) respectively. After the agar had solidified, the plates were incubated at 27° for 5 days, and the colonies counted. Although the colony counts varied between the 10 mice, the mean value represented the recovery of the indicator organisms from the mouse spleens at the predetermined intervals after i.v. injection. About 10^3 organisms was found optimal, because this number gave a recovery of approximately 100 colonies on each plate. When this procedure was applied to normal, SCY- or Killed-vaccine-immunized groups of mice, the mean value was less after 6 h compared with 3 h in the 222/+-immunized mice. This early drop between 3 to 6 h was considered to represent specific macrophage activity in 222/+-immunized mice and is expressed by the index: mean value of normal mice divided by mean value of 222/+-immunized mice. According to this procedure, this index was less than 2·0 up to the 8th day after 222/+-immunization, but it increased to greater than 2·0 after 9 days on repeated trials. On the other hand, this index never rose above 2·0 in killed vaccine- or SCY-immunized mice even after 14 days of immunization. ^{60}Co irradiation (400 rad) at the time of 222/+-immunization lowered this index to 1·0, but similar treatment (600 rad) at 14 days gave an index of 2·0. From these accumulated results, we concluded that the bactericidal activity of macrophages could be measured by SCFBT.

Measurement of delayed hypersensitivity (paraffin block method)

A centrifuged precipitate of a formalin-killed plague

vaccine (lot 56, produced by National Institute of Health, Tokyo, Japan) was used as the assay antigen. This liquid vaccine suspension was centrifuged and the supernatant discarded. One hundred mg of this packed antigen was emulsified into 10 ml melted paraffin (m.t. 36–38° prepared by mixing 4 volume m.t. 51–53° paraffin (Merck) with 13 volume liquid paraffin (Kokusan Kagaku Co. Ltd., Japan)) at 45°, at which temperature it remains liquid. Two tenths ml of this antigen-paraffin emulsion was injected intramuscularly (i.m.) into the femoral muscle of a mouse through a warmed syringe connected with a short needle, as used for the intracerebral injection of mice. After 24 h and 48 h, respectively, the mice were bled out and the site of injection containing the paraffin was cooled by gentle rubbing with dry-ice. When the paraffin had solidified and became visible as a white mass through the skin, an incision was made, the paraffin block was removed, and melted in 10 ml saline in a centrifuge tube, by warming at 45°. This was then centrifuged at 800 *g* for 6 min. During centrifugation, mouse cells mixed with or adhering to the paraffin block were released and precipitated and the paraffin solidified, forming a disc, which floated to the top. The paraffin was discarded and the packed cells resuspended in the saline. The cells were counted in a Coulter Counter model ZB1 (Coulter Electronics, Inc.) at the pore size of 4·92 μm.

These measurements were made on normal, 222/+-immunized and SCY-immunized groups of mice. Each group consists of 14 animals, 7 were bled 20–26 h after the injection of the antigen-paraffin mixture and the remaining 7 after 44–50 h. Values in each group were averaged to determine the degree of sensitivity. Similarly treated groups of mice were killed with ether at the corresponding times and haematoxylin-eosin stained sections of the antigen-paraffin injection site were examined to determine the leucocyte infiltration in response to the antigen.

Detection of serum antibody

Microagar diffusion tests were performed by the method of Wadsworth (1957). Serial 2-fold dilutions of acetone-treated bacterial extracts of each strain served as antigen. Serial 2-fold dilutions of non-heated serum, separated from the individual bleedings were examined for the formation of precipitin lines in various combinations with these dilutions of the antigen.

RESULTS

Protection of mice actively immunized with SCY and 222/+ against plague

Each of 20 SCY- and 222/+-immunized mice were injected s.c. with $5{\cdot}2 \times 10^5$ Yreka organisms (ca: 10^5 LD_{50}), as were the same number of non-immunized controls. The results are shown in Fig. 1; all control mice died within 10 days. Half of the 222/+-immunized group died within 10 days but the other half survived until the termination of the experiment (40th day, long term protection). In contrast, the superior short term protection afforded by SCY-immunization is clear. Few deaths occurred until the 21st day, but then 10 deaths occurred between the 10th and the 30th day resulting finally in less protection. However, no deaths occurred subsequently.

Passive protection of mice receiving SCY- and 222/+-immune serum against plague

Groups of thirty SCY- or 222/+- immunized mice were bled after their immunization programmes. A similar group of 30 non-immune mice were also bled. The separated serum of each group was pooled and 0·5 ml injected i.v. into each of 10 mice. Convalescent serum was also pooled and 0·25 ml injected i.v. into 10 mice (see Materials and Methods). One hour after injection these 4 groups of mice and a further 10 non-immune mice were injected s.c. with $1{\cdot}3 \times 10^6$ Yreka organisms. The results are shown in Fig. 2. Animals passively immunized with normal serum or anti-222/+-immune serum and unimmunized animals gave similar daily death rates. On the other hand, the two groups receiving anti-SCY immune serum or convalescent serum, survived longer. However, most mice of these groups, also died within 13 days of challenge, with only 2–3 survivals on the 16th day at the termination of the experiment. It was concluded that serum from SCY-immunized and convalescent mice contained protective humoral antibodies, but the serum from 222/+-immunized mice did not.

Measurement of bactericidal activity of macrophages (SCFBT)

Since the above results suggested a difference between short term protection following SCY-

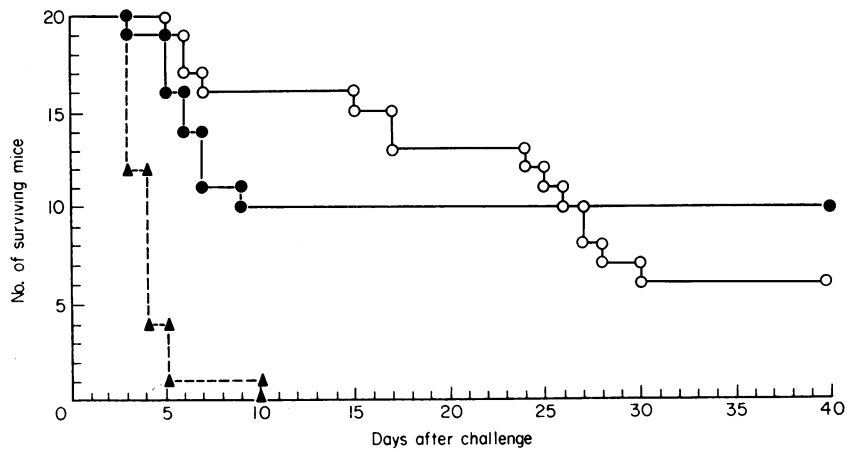

Figure 1. Protection resulting from active immunization of mice, showing survival of SCY-(○), 222/+-(●) immunized and non-immunized (△) mice, after s.c. challenge with $5{\cdot}2 \times 10^5$ organisms (approximately 10^5 LD_{50}) of a virulent plague strain Yreka.

immunization and long term protection resulting from 222/+-immunization, it was possible that this difference might be reflected in a particular organ responsible for the defence against pathogens. Accordingly, we developed a routine procedure for testing the bactericidal activity of macrophages (SCFBT), and applied it to SCY- and 222/+-immunized groups of mice, using normal mice as controls. These three groups, each consisting of 10 mice, were injected i.v. with $1{\cdot}1 \times 10^3$ SCY organisms

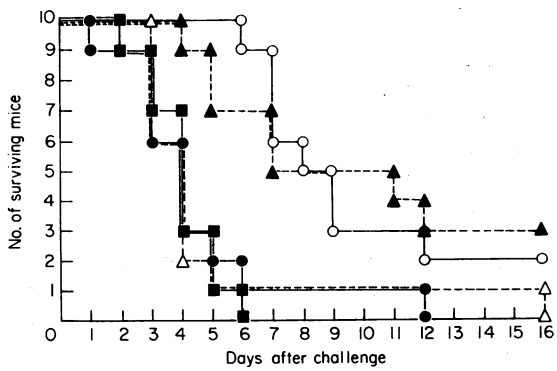

Figure 2. Protection from passive immunization of mice, showing survival of mice injected i.v. with: SCY-(○), 222/+-(●) immunized serum: convalescent serum (▲): normal serum (■): no serum (△), after challenge with $1{\cdot}3 \times 10^6$ organisms (approximately 10^5 LD_{50}) of the virulent plague strain Yreka.

14 days after immunization. The immunizing dose of SCY was $1{\cdot}6 \times 10^5$ organisms, while that of 222/+, was $2{\cdot}8 \times 10^2$ organisms. Hot agar suspensions of spleen cells were overlayed onto agar plates containing SM and CM between 3 h and 4 h 40 min after injection of the indicator SCY strain as already described (Materials and Methods). Aliquots of the supernatants after the removal of the spleen cells, were also mixed with hot agar plates. After 4 day's incubation at 27°, the number of SCY colonies growing on the plates showed significant differences between the 3 groups (Table 1).

Measurement of delayed hypersensitivity (paraffin block method)

Although the presence of delayed hypersensitivity in the 222/+-immunized group had been shown in our preliminary foot pad tests, direct counting of the leucocytes recruited around the reaction site should be a more reliable measure of delayed hypersensitivity. Accordingly, our paraffin block method was used to measure the degree of reaction in SCY-, 222/+-immunized and non-immunized mice. The results are shown in Table 2.

In spite of large standard deviations in the 222/+-immunized group, it is clear that more leucocytes were concentrated in the antigen-containing intramuscular paraffin mass than in the corresponding sites of SCY-immunized mice. That these reactions in the 222/+-immunized group are indicative of

Table 1. Measurement of bactericidal activity of macrophages (SCFBT)

Immunizing antigen	No. of colony forming units per mouse spleen		
	SCY	222/+	None
Cell fraction*	168·70 ± 62·52	93·40 ± 49·27	193·50 ± 98·00‡
Supernatant fraction	45·00 ± 25·30	28·80 ± 17·96	63·30 ± 31·16§
SCFBT index†	1·15	2·1	1·0
Index applied to supernatant†	1·4	2·2	1·0

* Values given are mean ± s.e. of colony forming units (CFU) of SCY, recovered from mouse spleen cell suspensions.
† See Materials and Methods.
‡ Significant difference among three groups within $P < 0·05$.
§ Significant difference among three groups within $P < 0·01$.

delayed hypersensitivity, is compatible with the histopathological findings where monocytes are predominant in the sections from the 222/+-immunized mice, whilst polymorph leucocytes are predominant in the sections from the SCY-immunized control mice (Fig. 3).

Detection of serum antibody

Serial 2-fold saline dilutions of serum samples, from a single mouse, belonging to the 222/+-, the SCY-immunized and the non-immunized groups, respectively, were examined for their specific antibody content against the serial 2-fold saline dilutions of acetone-treated plague antigen, using the microagar gel diffusion method. Although only a randomly selected single mouse serum from each group was tested, precipitin lines were formed only between the serum from the SCY-immunized mouse and the plague antigen containing wells.

DISCUSSION

Classical studies by Mackaness and colleagues (Mackaness, 1971) with *Listeria monocytogenes* have provided substantial evidence for cell-mediated immunity as a primary mechanism for acquired resistance against facultative intracellular bacterial infection. Confirmatory experimental data have been reported for the role of CMI in tuberculosis (Blanden, Lefford & Mackaness, 1969), brucellosis (Mackaness, 1964), salmonellosis (Blanden, Mackaness & Collins, 1966) and turalaemia (Kostiala, McGregor & Logie, 1975). The reason plague has been excluded from this group of infections seems to depend on the extremely acute lethal phase of human pulmonary plague, in which the extracellular growth of the bacteria results in septicaemia and death. It has been well documented that the fraction I 'envelope' antigen of *Yersinia pestis* is the major factor for immunizing man against plague infection

Table 2. Measurement of delayed hypersensitivity by paraffin block method

Time after antigen injection	20–26 h			44–48 h		
Immunizing antigen	SCY	222/+	None	SCY	222/+	None
No. of recruited leucocytes* ($\times 10^4$)	9·6 ± 4·7	64·2 ± 34·1	11·8 ± 7·2	14·4 ± 8·3	75·8 ± 63·5	20·5 ± 17·4

* Values given are mean ± s.e. of numbers of Coulter Counter counted leucocytes recovered from antigen-paraffin mixtures.

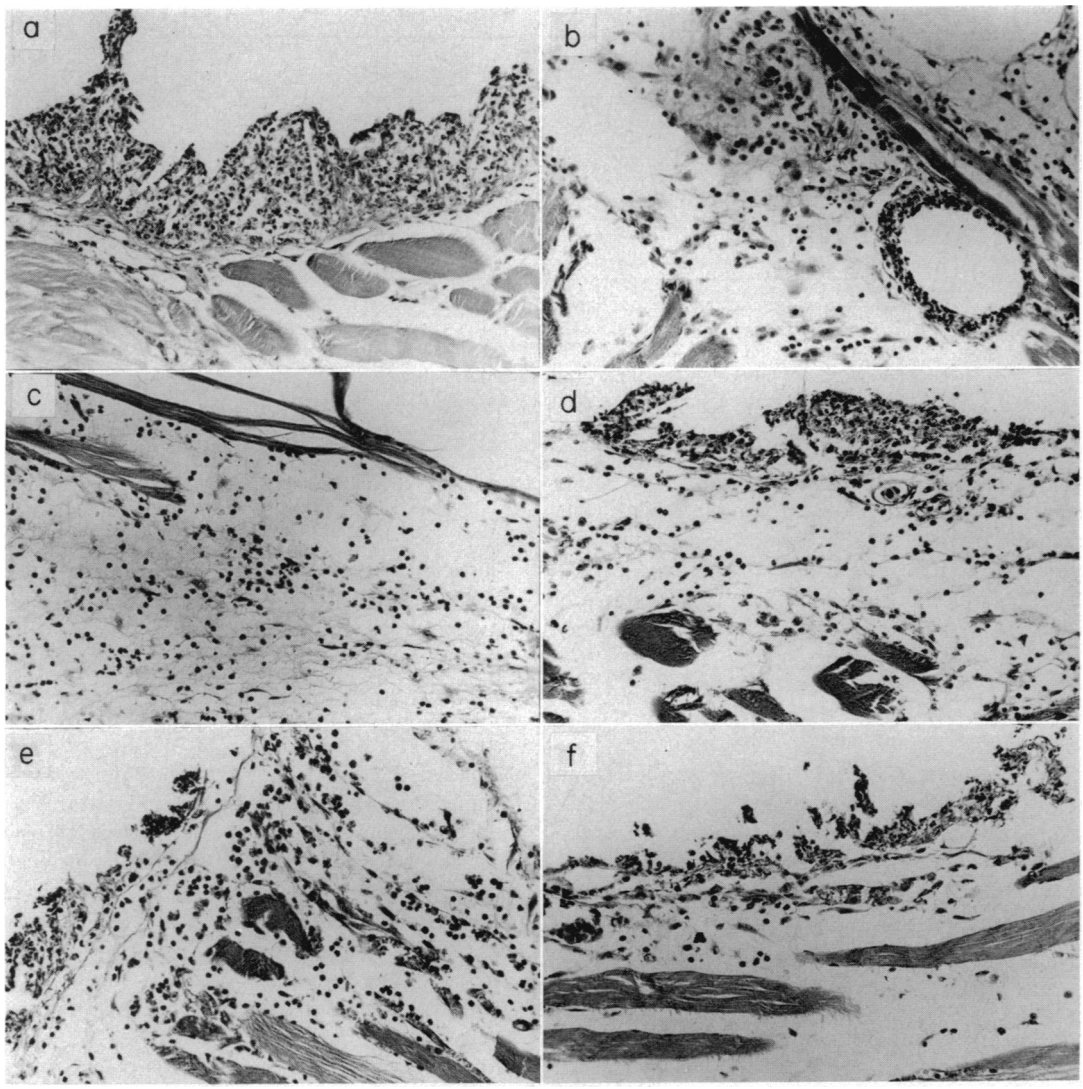

Figure 3. Femoral muscle sections of SCY- (a, b), 222/+- (c, d, e) immunized and normal (f) mice, taken 24 h (a, c, f) and 48 h (b, d, e) after injection of formalin-treated plague organisms and 0·2 ml melted paraffin mixtures (H & E, ×150).

(Meyer, 1953). V and W antigens (Burrows & Bacon, 1956) have been shown to confer protective immunity in mice by stimulating anti-V humoral antibodies (Lawton, Erdman & Surgalla, 1963).

Despite the circumstantial evidence for humoral protective antibodies, the production of allergic reactions in guinea-pig skins inoculated with living plague vaccine has been taken to indicate delayed hypersensitivity caused by CMI response to plague antigens (Korobkova, 1956). On the other hand, Cavanaugh & Randall (1959) pointed out the facultatively intracellular parasitism of flea-born plague bacteria (Thal, 1955) suggested the existence of another undetermined immunogen, which might conceivably promote cellular immunity.

Preliminary studies showed that guinea-pigs immunized s.c. with only 10^1 organisms of a living P− plague strain (EV) suspended in Blutal, were protected against challenge with even 10^7 LD_{50} doses of a virulent strain (Yreka). Positive skin reactions

of delayed hypersensitivity and macrophage migration inhibition tests, indicated the presence of CMI in these animals. Intravenous or intraperitoneal transfer of immune peritoneal cells protected the recipients significantly, against infection by the same route until the animals died from a graft versus host reaction. However, the plague murine toxin hampered the use of EV in a similar mouse experimental system. VW+ Y. *pseudotuberculosis* strain 222/+ suspended in Blutal was therefore used. The growth of Blutal suspended plague bacilli *in vivo* has already been reported (Tsukano, Yamamoto & Wake, 1972). Multiplication was localized within the injected area, and then the bacteria gradually disappeared. The iron chondroitin sulphate constituent of Blutal, has been reported to be phagocytosed by macrophages (Seno, Tanaka, Hirata, Nakatsuka & Yamamoto, 1975), and conceivably saturates the iron-binding proteins within macrophages resulting in accelerated intracellular bacterial growth. The strain 222/+ was selected because it was non-pathogenic for mice, and yet stimulated protective immunity against plague within 2 weeks of immunization (Fig. 1); it does not produce murine toxin. In the case of extremely large doses of virulent plague bacteria, organisms which escaped phagocytosis would multiply extracellularly within antibody-free spaces of 222/+- immunized mice, and such mice would occasionally die in a short time, seen in Fig. 1. If the animals successfully phagocytosed all invading bacteria, they survived until the termination of the experiment (40th day), because the phagocytosed bacteria were completely killed within activated macrophages. This view has been supported by Zinkernagel (1976), who agreed with our explanation that spleen macrophages, activated by CMI mechanisms, lowered the viable number of the indicator strain injected into the 222/+-immunized mice in our technique for measuring the bactericidal activity of macrophages. It was concluded that it would be reasonable to utilize SCFBT as an indicator of CMI.

In the paraffin block method, positive delayed hypersensitivity could easily be distinguished, because the inflammation produced remarkable swelling of the antigen-containing mouse thighs. However, Coulter Counter counting of recruited leucocytes gave a more objective numerical value, when the technique of paraffin-block sampling was perfected. It was noteworthy that only 222/+-immunized mice gave significantly higher values of leucocyte counts indicating the CMI nature of the protective immunity in this group. This study adds more evidence for the existence of CMI providing the long term protection against plague. Furthermore, the immunizing strain 222/+ produces VW but not F1. Further work will be needed to identify the responsible immunogen as VW, because the third immunogen pointed out by Thal (1955) has not been excluded from our 222/+ strain, despite the preliminary result that a VW-lacking Y. *pseudotuberculosis* strain 14I and our VW-less Y. *pestis* mutant strain MII40 could stimulate only the smallest degree of protection in similar experiments. In contrast, the VW-lacking, F1-producing Y. *pestis* strain SCY significantly protected the mice from deaths but only during the initial phase of the virulent challenge (short term protection, Fig. 1). The role of humoral opsonizing antibodies is apparent, because the passively transferred serum, separated from SCY-immunized mice, significantly prolonged the survival periods of the recipient mice (Fig. 2).

It is conceivable that the intracellular killing activity in these mice was not as complete as in 222/+-immunized mice, because deaths occurred frequently until the 30th day after virulent challenge in the actively immunized group (Fig. 1), and 12th day in the passively immunized group (Fig. 2). Almost all of the non-immunized mice were killed within 5 days by this virulent challenge, so some of the phagocytosed bacteria could persist, presumably alive, within macrophages from 1 to 4 weeks, and multiply to destroy the macrophages and kill the host animal. This non-activation of macrophages or lack of CMI in SCY-immunized mice was well reflected in the measurements of the bactericidal activity of macrophages (Table 1) and of delayed hypersensitivity (Table 2). These results suggest some failure in the traditional method used for measuring cell-mediated protection against bacterial infections, in which the number of viable bacteria in a selected organ is counted at a certain interval after virulent challenge, and compared with those in the control groups. If this method had been used in our experiment, for example, 10 days after virulent challenge, the SCY-immunized group would give a lower number of viable bacteria than the 222/+-immunized group, and the difference between short term and long term protection would be missed. Despite the present evidence for the role of CMI in immunity to plague, the direct demonstration of im-

mune lymphocyte transfer is desirable to confirm the theory. The mouse strain dd/Y, used in this present study, is inadequate for live-cell-transfer experiments, because it is an outbred strain. Successful demonstration of immune lymphocyte transfer accompanied with the acquisition of protective immunity by C57BL/6J mice from those of the same strain, immunized with 222/+, has now been achieved and will be published shortly.

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