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MECHANISM OF FLAGELLAR VACCINE PROTECTION RELATED TO PSEUDOMONAS PATHOGENESIS IN TRAUMA BURNS

Annual and Final Report

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Flagella protein has been purified by differential centrifugation and by passage through molecular sieving columns with addition of EDTA and dexoxycholate. By this treatment, lipopolysaccharide contamination was routinely reduced to insignificant amounts. Initial amino acid analysis has shown the absence of several amino acids. N-terminal sequence analysis showed that a common sequence existed in all antigen types examined. Flagellin from homogenous b-type flagella showed a M_r of 53 kDa, whereas heterogenous a-types were from 45 to 52 kDa. Other *Pseudomonas spp.* have different molecular weight flagellins and possess species specific antigens. An ELISA was developed which distinguished a-types (a_0, a_1, a_2, a_3, a_4 combinations) from b-type antigen in the nanogram-microgram range in various flagella preparations or on formalized whole cells. A strongly reactive, common a_0 epitope is identified by ELISA in standard strains or in clinical isolates. Using isogenic mutants we have demonstrated that loss of flagellum or motility results in a significant loss of

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virulence in *P. aeruginosa*. A flagellum plus revertant regained virulence. Isolated flagellar preparations have provided active protection in a burned mouse model. Passive protection with anti-flagellar sera (anti-LPS-free) showed specificity for flagellar type and gave significant protection in two different burned mouse models. The protective antibody is IgG. Again cross-protection among a-types was obtained. These preparations have been used in Western-blots to identify flagella antigen. Flagellar antibody has been shown to stimulate opsonophagocytosis. This phagocytosis was flagellar antigen specific and confirmed the presence of a dominant a_0 epitope in the heterogenous a-types. We have concluded from this research that flagella antigens show excellent potential as vaccine components and that two antigens, a-type and b-type would need to be included.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW) Publication No. (NIH) 78-23, Revised 1978.

TABLE OF CONTENTS

The following sections summarize the highlights our research contract work during the period 1982 to 1986. Additional discussion will emphasize research accomplished during 1987 to the termination of contract.

I. Research Period, 1982 to 1986

A. Purification and Characterization of Flagellar antigen from Psuedomonas aeruginosa

1. Isolation and purification

The first phase of the purification scheme as initially developed (see Phase I, Flagellar Antigen Isolation/Purification Scheme, Appendix pg 13) involved the means to obtain partially purified flagellar antigen preparations (FLAg) from large batches of *Pseudomonas aeruginosa*. As observed initially, a key was to grow *P. aeruginosa* at 30°C in a single mineral-salts medium with succinate as the carbon source. These conditions resulted in the least amount of contamination of flagellar preparations with respect to other proteins and lipopolysaccharides (LPS).

Flagella are removed from bacteria by shearing in a blender under carefully controlled conditions. A partially purified preparation of FLAg are obtained by differential centrifugation (1, 3). At this stage, preparations contain from 5 to 15% LPS.

Phase 2 of the purification involves two column steps. The primary column step requires a molecular filtration column used for very large molecular weight separations. Importantly, addition of EDTA (0.1 M) and deoxycholate (0.1 %) to the FLAg and passage through a Sephacryl 300 column (equilibrated with 0.2% deoxycholate and Tris buffer) removes and retards LPS while the purified high molec ar flagella protein peaks at

the void column. A second Sephadex G-25 column is used primarily to removed residual LPS and deoxycholate from the pooled flagella fractions.

Currently, from 12 L of medium, we obtain approximately 20 mg partially purified FLAg after column treatment. By chemical, rabbit pyrogenicity, or Limulus analysis, LPS is shown to be reduced in purified preparations to 0.1 - 1.0 % LPS.

2. Antigen Properties

a. Isolates, both standard laboratory and clinical, exhibit either a homogeneous b-type or a heterogenous a-type flagellin (2, 3, 4). Fifty to sixty percent of isolates are b-type. The remainding a-types exhibit heterogeneity in subtypes with possible combinations of the subantigens $a_0a_1a_2a_3a_4$. A recent important finding was that all a-types contain a cross-reactive a_0 epitope as demonstrated by ELISA (4), opsonophagocytosis (5), and burned mouse protection experiments (Montie et al, unpublished data). Another finding was that molecular weights distinguish the two groups of flagellins. The b-types are 53 kDa while the a-types vary from 45 to 52 kDa depending on the subantigen compositions (3). In comparison, we have found that *Pseudomonas cepacia* posseses two types of flagellin that also are strain specific antigenically (6). Type I is 31 kDa and type II showed two closely paired bands of 44 and 46 kDa. Some heterogeneity existed in the type II group perhaps analogous to a-type *P. aeruginosa*. However, we have not pursued this latter aspect in detail.

b. Amino acid analysis of the various *P. aeruginosa* flagellins have shown some marked similarities as well as differences which

would account for the molecular weight differences observed in gel analysis (Table 1, Appendix). Notably, four amino acids are missing: proline, methionine, cystine, and histidine. In all strains except 170018, tyrosine is at very low levels and phenylalanine, although at higher levels, apparently has not replaced the tyrosine to any extent. This low level of aromatic amino acids accounts for the lack of absorption in the 270-280 range with a shift in peak absorption to 260-265 nm. We have elected therefore to use globulin as our standard protein in routine protein determination.

In collaboration, N-terminal sequences for a number of flagellins (a- or b-types) were found to be the same up to nine amino acids sequenced. These data suggest that this portion of the flagellin functions in some common structural mode such as polymerization recognition.

3. Antigenicity

Initial assays performed for antigenicity involved slide agglutination reactions using suspensions of bacteria (1, 3). In general, results were in agreement with those of Ansorg (2) who supplied the initial standard strains used as a basis for comparisons. Ansorg (2) used indirect immunofluorescence to distinguish the a-subtypes. The a-or b- types could be identified in most cases by the slide agglutination technique, but the insensitivity of the method made typing difficult particularly of a number of a-types. An ELISA was developed using a preoxidase conjugate secondary antibody. In this technique using our standard

adsorbed rabbit antisera, we were able to show that any anti-LPS present was reduced to extremely low levels (7). From continued developments it was demonstrated that formalized whole bacteria could be used as a specific source of antigen and that a common dominant subantigen was present in all a-types, Table 2 (4, 9). We use the designation a_0 first used by Ansorg to indicate this common epitope. An additional advantage of the ELISA than is that it allows for quantitation of the strength of unknown flagellar antibody or antigen. A relative value of the endpoint titer or plot of the titer data can be compared or the total adsorption read at 540 nm may be used for semi-quantitative comparisons.

B. Virulence studies

Initial observations utilizing some common laboratory strains indicated that flagellar or motility differences might help to explain some loss of virulence in an invasive burned-mouse model (9). A more detailed examination using an isogenic mutant of strain M-2 (M-2, Fla$^-$) showed this strains to have lost up to five logs in virulence (lethal dose) (10). A revertant isolate regained full virulence with almost all isogenic mutants composed (see below). Isogenicity was established with regard to growth rates, antibiotic and biochemical responses, O antigen and in some cases selected virulence factors exotoxin A and proteolytic activity. A series of AK mutants (obtained from Dr. Andy Koprinski) were also compared and tested versus the parent PAO1. Motility in these strains was deficient because of lack of a flagellum, but other physiological characteristics were normal (11). A third pair of strains were the MT 1200 strains obtained from Dr. Tsuda. The mutant here (1200-80) possessed a strain antigenically active flagellum. The latter strain was

unique in its Mot⁻ characteristic apparently due to the lack of the normal, wavey filament structure. Using the subcutaneous inoculation according to the model of Steritz and Holder (12), a decrease of from 3 to 5 logs in virulence was recorded for the Fla⁻ strains and 2 to 3 logs in lethal dose for the Mot⁻ strain (Table 3, appendix). Although not as sensitive because of its requirement for increased bacterial dose, topical applications were used for comparison (Table 4, appendix). Using the alcohol-flame wound and topical application, the M-2 Fla⁻ mutant was up to 100 fold less virulent than the wild type. Similarly, in a scalded burn mouse model (13), virulence was decreased, although to a smaller extent only 75% in the M-2 mutant (Table 4).

Decrease in virulence in non-motile strains (Fla⁻ or Mot⁻) was reflected in lack of invasiveness demonstrated in tissue colonization assays. Typically, inoculation of bacteria into the burn wound results in rapid skin colonization of both wild type and mutant strains. However, the major difference occured in the invasive potential of the organism (11). Within hours, wild type bacterial counts increased in the blood stream and internal organs such as the liver (Figure 2, appendix). In motility mutants, the bacteremic stage is severely decreased with virtually no bacterial counts in the blood fraction and a reduction in liver counts of five logs cfu (Figure 2 or 3, appendix).

C. Vaccine Protection Experiments

A series of experiments have been performed using flagellar antigen or hyperimmune rabbit sera to protect against burn wound infection. Active protection experiments were originally carried out in collaboration with Dr. I. A. Holder (Shriners Burn Institute and University of Cincinnati) (1, 14). Although these preparations are contaminated with lipopolysaccharides, only

1.0 ug were used in a single dose fourteen days prior to challenge. Data indicated that protection was complete (100%) and was specific for flagellar antigen. Only bacteria agglutinated by specific antiflagellar serum were protective against by the same antigen. A PA103 (Fla^-) flagellar extract (negative control) gave no protection. Heated bacteria did not agglutinate with anti-flagellar serum. Repetition and extension of these experiments in our laboratories showed that 1.0 ug flagellar antigen protected against challenge with 4.6×10^5 M-2 CFU (15). This challenge in the flame-burn model was equivalent to protection against 86,000 LD_{50} doses (15). In other experiments, heating of the flagellar antigen preparation destroyed both the majority of the antigenicity and a protective capacity. These results emphasized again the importance of the protein flagella antigen in the preparations.

Another approach with more direct application for clinical use is passive immunization. A number of experiments therefore were carried out using hyperimmune rabbit anti-flagellar serum.

The adsorbed rabbit antisera used was characterized and shown by ELISA to contain primarily anti-flagellar IgG which type-specifically inhibited motility (7). Reaction with LPS antigen was zero showing that background O-type antibody was not a contributing factor. In ELISA studies, it was demonstrated that antibody was H-type specific for a-type or b-type flagellar antigen.

This rabbit antiserum was used in passive burned-mouse protection studies (7). The antiserum was diluted 100-fold and 0.5 ml injected prior to challenge or coincident with challenge and in some cases one day post-burn. In Tables 5 and 6 (appendix), we summarize data where antibody injection was

one day prior to burning. In every case, protection coincided with matching FLAg type. For example, challenge with a clinical burns isolate SBI-I, determined to be a b-type, was protected against with M-2 b-type antisera. Again, no association was seen between the O-type of the challenge organism and protection (or lack of) with a given antiserum. Similarly (data not shown), a-type antiserum only protected against a-type challenge. Subsequently, we have shown that immunization on day of challenge and post-challenge also produces significant protection.

In a control study, antiserum was adsorbed with an isogenic Fla^+ or a Fla^- strain and the two samples compared for protective capacity. Protection occurred only in the antiserum absorbed with Fla^- cells. This result indicated that specific removal of flagellar antibody coincided with loss of protective capacity.

In these same studies (7), mice were challenged topically. Although total protection was not achieved, delay in time till death was seen in flagellar anti-serum treated mice. Subsequently, we showed that a high level of protection from topical challenge could be achieved, (Table 4, Montie *et al* unpublished results). We have since concluded that in the initial experiments we were challenging with an unreasonably high dose.

Consistent with the lethality-protection studies were results from experiments that showed inhibition of *P. aeruginosa* invasion by flagellar antiserum (7). Bacterial counts of tissues following challenge revealed patterns similar to those using Fla^- mutants. Animals injected with anti-FLAg serum showed reduced skin counts, 2 logs lower than non-immunized mice at 24 hr post-challenge and post-immunization. No bacteria were detected in liver and blood counts of immunized animals at 24 hrs. After eight days, liver

counts never rose above 10^3 CFU in immunized animals, and they remained lower (10^1 CFU) if 2 immunizations were used.

D. Phagocytosis Studies

As noted in the 1987 Annual Report, phagocytosis studies were initiated and it was demonstrated that hyper-immune rabbit anti-flagellar serum was opsonic as assayed by an indirect CFU phagocytosis assay. Subsequently, additional results showed that b-type serum only served as an opsonin for b-type organisms. Similar results were obtained for strain SBI-N, a human burn isolate (a-type subantigen unknown), using 1210 antiserum ($a_0a_1a_2$) (5). These results were in agreement with the vaccine protection results and ELISA studies which showed the specificity of antibody for the homologous antigen, a or b.

II. Final Research Period, 1987 to April 1, 1988

A. Purification and characterization of flagellar antigen

During 1987 studies were continued on purification and analysis of FLAg. As we mentioned in our March 1987 report, we had obtained definitive ELISA data using isolated FLAg or intact cells as antigen course; that a common a_0 epitope was present in all a strains characterized. Although these flagellar types generally contain additional subantigens, the a_0 epitope exhibited sufficient dominance to give strong cross-reactivity (4). With the use of a "Phast gel apparatus" (Pharmacia), FLAg preparations could be assayed and stained by SDS-PAGE in a computer programmed time of 60 min. These mini gels can be dried and permanently stored. A method was developed for FLAg transfer by Dr. Helen Sellin by pressing these gels against nitrocellulose paper (cf Pharmacia protocols). The transferred antigen then could be immunoblotted using goat anti-rabbit IgG conjugated to peroxidase as in the ELISA method.

Some important Western blots results were as follows. Material taken from the first peak (Peak I) emerging from the EDTA-DOC molecular sieving column gave a single band with either whole killed b-type antisera (PJ108 or M-2) using crude M-2 antisera or antisera made to purified material. On the other hand, crude M-2 antigen preparations gave a second more mobile band that was not seen in more purified preparations. Several bands were seen in M-2 or PAO-1 LPS preparations when reacted with anti-LPS antisera. These bands did not correspond to the single band appearing in the flagella purified preparations. These results confirmed again the value of the above discussed purification procedures in their demonstration of the removal of impurities such as LPS.

Phagocytosis Studies

The preliminary opsonophagocytosis results showed that flagellar antibody acted as a specific opsonin, but were based entirely on the indirect method. This method involves measurement of the number of bacteria removal (CFU) from supernatants. To sensitize this approach, strain PAO1 was radiolabelled with ^{3}H-thymidine in mineral salts 0.4% sodium succinate medium for 15h at 37° (16). Well-washed bacteria were opsonized by the addition of 0.1% serum and incubation for 30 min as previously described (5). The reaction suspension was centrifuged at 400 x g for seven min to separate PMNs with cell-associated bacteria from non-cell associated bacteria. A control tube was centrifuged at 4000 x g for 15 min to obtain total bacterial counts. Pellets were suspended in Bray's solution and counted. Counts were adjusted for background activity. Percent leukocyte-associated bacteria was determined, and expressed relative to total bacteria available for phagocytosis and counts obtained of bacteria opsonized in normal rabbit serum. Internalization of opsonized bacteria by phagocytes was also visualized by electron microscopy.

By measuring counts directly in the pellet fraction we verified results reported which utilized the indirect method (5). That is, that hyperimmune rabbit antibody was opsonic (enhanced phagocytosis 3 to 4 times) and specific for the flagellar type (a or b). An extended second finding of significance was that regardless of the a subtype ($a_0a_1a_2a_3a_4$ and any combination), the a_- or common antigen present in four different a strains tested, reacted opsonically with standard a-type $a_0a_1a_2$ anti-1210 flagellar antibody. These data again add support for the vaccine potential of the flagella antigen, because only two strongly reactive antigens (antibodies) need be administered to cover all clinical isolates.

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FIGURE I. FLAGELLAR ANTIGEN ISOLATION/PURIFICATION SCHEME

Phage 1 - Crude (Unpurified) Flagella Isolation

1. Inoculate 60 ml Minimal Media (MM) plus appropriate carbon source(s) with 1 ml refrigerator stock culture. Incubate at 37°C for 20 hrs or until O.D. is greater than 0.75 A.
2. Inoculate each of 12 1L flasks containing 500 mls MM with 5 mls overnight culture. Incubate at 30°C for 24 hrs or until O.D. is greater than 0.5 A.
3. Concentrate cells into 6, pre-weighed centrifuge tubes by successive spins at 5000 X g for 15 minutes. Spin at 4°C.
4. Resuspend cell pellets in 0.01 M potassium phosphate buffer (200 mls) with phenylmethylsulfonylfluoride (PMSF) at 0.1 mM. Use buffer at room temperature to prevent cold shock.
5. Shear flagella in Waring Blender for 30 second at LOW speed.
6. Centrifuge the sheared cells suspension for 30 minutes at 5000 X g (4°C). Pour off and save flagella-bearing supernatant. Weigh and discard cell pellet.
7. Divide supernatant into six steel centrifuge tubes kept on ice. Spin at 16,000 X g (4°C) for 15 minutes. Remove and save supernatant, discard pellet.
8. Ultracentrifuge supernatant at 40,000 X g for 3 hrs. Use Type 35 rotor and capped tubes (35 mls/tube).
9. Drain tubes and resuspend pellets with wash from pipette. Use 20 mls cold Column B Buffer (Column B Buffer = 0.1% sodium azide, 30 mM Tris-HCl, pH 8) to resuspend pellets and 5 mls to wash tubes.

10. Dialyze overnight with gentle stirring in cold room against 3L cold dH_2O. Change wash, and dialyze again for 6 hrs. Lyophilize and weigh samples.

Phase 2 - Flagellar Antigen Purification

11. Redissolve lyophilized prep in 2-3 mls sample buffer (0.1% sodium azide, 15 mM Tris-HCl, pH 8, 1 mg/ml deoxycholate, 0.1 M EDTA, 0.1 mM PMSF).
12. Run through Column A (Sephacryl GF-2000 packed in an 80 cm x 1.5 cm glass column) at pump setting 2.5, 5 mV chart sensitivity, and 1.0 absorbance setting. Run with Column A Buffer (0.1% sodium azide, 30 mM Tris-HCl, pH 8, 2 mg/ml DOC)
13. Load whole peak fraction onto Column B (Sephadex G-25 packed in a 30 cm x 1.5 cm glass column). Run at settings listed in (12).
14. Collect peak fraction, dialyze overnight in cold room against 3L dH_2O. Change wash, and dialyze again for 5 hrs against cold dH_2O.
15. Lyophilize fractions. Weight final FAg product. Store in a capped vial at -70°C.

Table 1: Antigenic components and molecular weights of flagella of some representative antigen-characterized (standard) and unknown *Pseudomonas aeruginosa* strains.

Strain designation	Antigenic component(s)[a]	Flagellin mol. wt.
PJ108	b	53,000
19660	–	52,000
5939	a_0, a_3	52,000
5933	a_0, a_1, a_2	51,000
1210	a_0, a_1, a_2	51,000
7191	a_0, a_1, a_2	51,000
5940	a_0, a_2	47,000
170018	a_0, a_3, a_4	45,000
GNB-1	–	49,000
3598 (F)	–	48,000
3614 (F)	–	48,000
86-f (CF)	–	48,000
572b (CF)	–	48,000

[a]These antigenic types were differentiated by the indirect fluorescent-antibody and agglutination techniques (6).

GNB, general clinical non-burn isolate; F, folliculitis isolate; CF, cystic fibrosis isolate.

Table 2. Determination of *Pseudomonas aeruginosa* flagella type in bacterial preparations by ELISA.

Flagella type			Cross reactivity (antisera)[a]		
Strain designation	Slide agglutination	ELISA	PJ108 (b)	1210 (a_0, a_1, a_2)	170018 (a_0, a_3, a_4)
PJ108	b	b	++++	+	+
1210	a	a	+	+++	+++
170018	a	a	–	+++	++++
19660	b	b	++++	+	+
5939	NR	a	+	+++	++++
5933	a	a	+	+	++++
7191	a/b	a	+	+++	+++
5940	a	a	+	+++	++++
170002	NR	a	+	+++	++++
170012	NR	a	–	+++	+++
GNB-1	a	a	+	+	+++
3592	NR	a	+	+++	+++
3598 (F)	NR	a	+	+++	+++
3614 (F)	a	a	+	+++	++++
WR-5	NR	a	+	+	++++
86-f (CF)	a	a	+	+++	+++
572b (CF)	NR	a	–	+	+++
CF-LL	NR	a	+	+++	+++

[a]Cross-reactivity is based on ELISA titers: < 1000 (–), 1,000 – 16,000 (+), 32,000 – 128,000 (+++), 256,000 – 512,000 (++++).

GNB, general clinical non-burn isolate; F, folliculitis isolate; CF, cystic fibrosis isolate; NR, not reactive.

Table 3. *Virulence, as expressed in s.c. mouse challenge, of P. aeruginosa strains differing in the Fla or Mot phenotype*

Strain	Challenge inoculum (c.f.u.)	Cumulative mortality (died/total inoc.) day 7 postburn
M-2 (Fla$^+$ Mot$^+$ parent)	4.8×10^{7}	10/10
	4.8×10^{6}	9/10
	4.8×10^{5}	3/10
M-2 Fla$^-$ (Fla$^-$ Mot$^-$ mutant)	1.4×10^{7}	5/5
	1.4×10^{6}	4/5
	1.4×10^{5}	3/5
	1.4×10^{4}	1/5
	1.4×10^{3}	0/5
PAO1 (Fla$^+$ Mot$^+$ parent)	5.3×10^{3}	5/5
	5.3×10^{2}	4/5
	5.3×10^{1}	2/5
AK1152 (Fla$^-$ Mot$^-$ mutant)	8.3×10^{6}	1/5
	8.3×10^{5}	0/5
AK1153 (Fla$^-$ Mot$^-$ mutant)	1.1×10^{7}	1/5
	1.1×10^{6}	0/5
MT1200 (Fla$^+$ Mot$^+$ parent)	6.2×10^{5}	10/10
	6.2×10^{4}	8/10
	6.2×10^{3}	5/10
	6.2×10^{1}	1/10
MT1200-80 (Fla$^+$ Mot$^-$ mutant)	9.4×10^{4}	0/10
	9.4×10^{3}	0/9
	9.4×10^{2}	0/10
Controls	—	0/15

Table 4. *Virulence, as expressed in topical mouse challenge, of P. aeruginosa strains differing in the Fla or Mot phenotype*

Model	Strain	Challenge inoculum (c.f.u.)	Cumulative mortality (died/total inoc.) day 7 postburn
Alcohol-flame	M-2 (Fla$^+$ Mot$^+$ parent)	2.75×10^{9}	10/10
		2.75×10^{8}	8/10
		2.75×10^{7}	6/10
		2.75×10^{6}	4/10
	M-2 Fla$^-$ (Fla$^-$ Mot$^-$ mutant)	2.13×10^{9}	4/10
		2.13×10^{8}	4/10
		2.13×10^{7}	0/9
Scald	M-2 (Fla$^+$ Mot$^+$) parent	3.10×10^{7}	8/8 (8/8)*
	M-2 Fla$^-$ (Fla$^-$ Mot$^-$) mutant	3.60×10^{7}	6/8 (2/8)*
	MT1200 (Fla$^+$ Mot$^+$) parent	2.60×10^{9}	5/8
		2.60×10^{8}	1/6
	MT1200–80 (Fla$^+$ Mot$^-$) mutant	1.50×10^{9}	0/8
	Controls	—	0/11

* Values in parentheses are those obtained on day 2 postburn

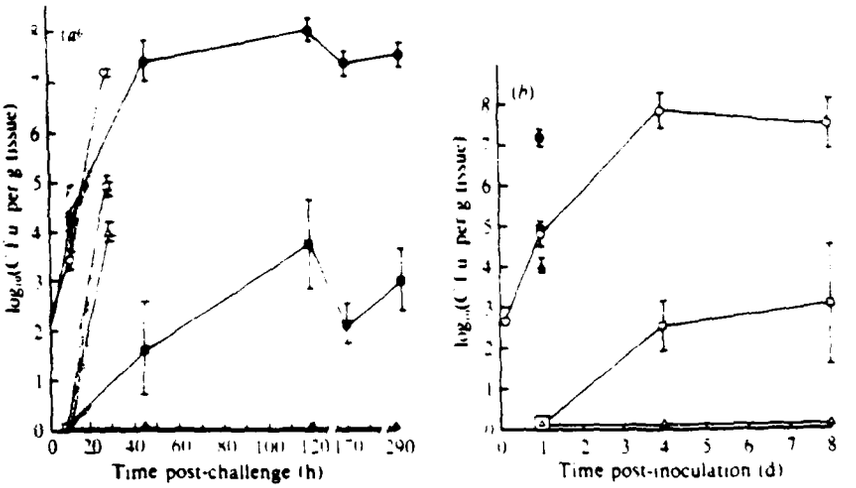

Fig. 2. (*a*) Bacterial counts of skin (○, ●), liver (□, ■) and blood (△, ▲) after s.c. challenge of mice with strain M-2 Fla$^+$ (open symbols) or M-2 Fla$^-$ (filled symbols). Data are expressed as means of values from three mice ± standard error of the mean. Earlier skin counts (M-2 Fla$^+$) from a similar experiment are: 8 h, 3×10^3 c f u g^{-1}; 16 h, 5×10^6 c f u g^{-1}; 22 h, 10^7 c f.u. g^{-1}. (*b*) Data from tissues of mice that received b-type anti-flagellar serum at day 0 and were challenged s.c. at day 0 with homologous strain M-2 (Fla$^+$) ○, Skin, □, liver, △, blood, filled symbols show counts from control mice that did not receive antiserum.

TABLE 5. Cross-challenge using *b*-type antisera and challenging with flagellar *a*- or *b*-type organisms

Mice*	Challenge (cfu)	Challenge strain H-type	Challenge strain O-type	Mortalities (total inoc.)
Noninjected	M-2(1.4×10^2)	*b*	(2), 5†	8(8)
	SBI-I(4.0×10^4)	*b*	10	7(8)
	1210(7.9×10^1)	*a*	6	8(8)
NRS administered‡	M-2(1.4×10^2)	*b*	(2), 5†	8(8)
	SBI-I(4.0×10^4)	*b*	10	7(8)
	1210(7.9×10^1)	*a*	6	8(8)
	GNB-I(4.0×10^4)	*a*	6	8(8)
Injected with M-2 H antisera	M-2(1.4×10^2)	*b*	5	0(8)§
	SBI-I(4.0×10^4)	*b*	10	0(8)¶
	1210(7.9×10^1)	*a*	6	8(8)
	GNB-I(4.0×10^4)	*a*	6	7(8)

*Antisera (100-fold diluted, 0.5 mL, i.p.) injected 1 day prior to burning.
†Subtype 5 was the primary reactive antigen.
‡NRS, normal rabbit serum.
§$P = 0.0008$ (significantly different at $\alpha = 0.05$ level).
¶$P = 0.0078$ (significantly different at $\alpha = 0.05$ level).

TABLE 6. Cross-challenge using 1210 *a*-type specific antisera and challenging with flagellar *a*- or *b*-type organisms*

Mice*	Challenge (cfu)†	Challenge strain H-type	Challenge strain O-type	Mortalities (total inoc.)
Noninjected	M-2(1.54×10^2)	*b*	(2),5	7(8)
NRS administered	M-2(1.54×10^2)	*b*	10	7(8)
	1210(1.09×10^2)	*a*	6	8(8)
Injected with 1210 antisera	M-2(1.54×10^2)	*b*	(2),5	7(8)
	1210(1.09×10^2)	*a*	6	1(8)‡

*Antisera (100-fold diluted, 0.5 mL, i.p.) injected immediately postburn.
†Subcutaneous.
‡$P = 0.00567$ (significantly different at $\alpha = 0.05$ level).

TABLE 7. FLAGELLAR ANTIGEN DEPENDENT CROSS-PROTECTION AFTER TOPICAL CHALLENGE USING a-TYPE FLAGELLAR ANTISERA[a]

CHALLENGE STRAIN	INOCULUM (CFU)	IMMUN	H TYPE	O TYPE	DEAD/ INOCULATED
7191	2.8×10^8	-	a	1	3/3
SBI-N	3.3×10^8	-	a	ND	3/3
	7.3×10^8	-	"	"	4/4
M2	1.0×10^7	-	b	5	4/4
	1.0×10^8	-	b	5	4/4
7191	2.8×10^8	+	a	1	0/6
SBI-N	3.3×10^8	+	a	ND	0/6
M2	4.1×10^8	+	b	5	6/6

[a]Strain 1210 antisera (a-type = $a_0a_1a_2$) and O type is 5. Antisera was adsorbed for O antibodies.

[b]Based on ELISA typing using adsorbed antisera. Strains 7191 and SBI-N reacted positively with 1210 ($a_0a_1a_2$) and 170018 ($a_0a_3a_4$).

[c]Based on Difco typing using slide agglutination.