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STUDIES ON LISTERELLA MONOCYTOGENES

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I. THE EFFECT OF VARIOUS KILLING AGENTS ON THE FLAGELLAR ANTIGENS

Among the many studies of the effect of various chemicals on serological specificity are few directed at the alteration of the specific character of the individual antigens of the bacterial cell, although there have been many investigations of the effect of preservatives on *in toto* serological activity. Where individual factors have been studied, the approach has ordinarily been to groups of antigenic factors, such as the flagellar and somatic components, or to isolated chemical entities, such as the specific carbohydrates of pneumococci. In the first instance the inactivating effect of prolonged heating or alcohol treatment and the preservative action of such agents as formaldehyde on the flagellar components are well known. Many bacteriostatic agents have been used as preservative agents for bacterial vaccines, and while those in common use appear to have little deleterious effect on the ability of the vaccines to elicit protective antibodies, protection and full expression of antigenicity are not necessarily synonymous.

Although minor deficiencies in antigenicity may not impair the protective capacity of vaccines, they are definitely objectionable in antigenic analysis of bacteria. Killing agents and preservatives for suspensions to be used in this work must, therefore, alter the specificity of bacterial antigens to a minimal degree. Ungar and his associates (1) found little difference among heat, phenol, formalin, merthiolate, acetone, and chloroform as killing agents for organisms of the *Salmonella* group, although the slight variations in agglutinating capacity which did occur were consistent and led the authors to conclude that phenol, merthiolate, and chloroform caused the least alteration in immunogenic constitution. It may be significant that 0.5 per cent phenol was used as a preservative for all vaccines following washing to remove the killing agent. Vinogradova (2), similarly working with a *Salmonella* strain, found phenolized vaccines somewhat more protective than heat-killed. On the other hand Kreuger and Nichols (3) noted that undenatured staphylococcal antigens underwent marked denaturation when treated with merthiolate or phenol in the usual concentrations of preservative. Such denaturation would be expected to alter the serological specificity of the antigens although considerable protective capacity would probably be retained.

Because large volumes of bacterial suspensions were needed for experiments in antigenic analysis with *Listerella monocytogenes* and necessarily had to be kept for considerable periods, an investigation of the effect of selected killing agents on the antigenic structure of these organisms was undertaken.

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Paterson (4) has postulated four flagellar components in various strains of *Listerella monocytogenes* distributed among three groups as follows: Group 1, components AB; Group 2, components ABC; and Group 3, components BD. Factor "B" is common to all groups and is the major component in activity. Results obtained in this laboratory, as yet unpublished, indicate that the structure of the group is somewhat more complex than conceived by Paterson, but the effect of the various killing agents was studied only with respect to these clearly defined components.

METHODS AND RESULTS

The strains of *Listerella* used were selected for their known antigenicity and were constantly checked for stability with respect to motility and colonial morphology. Motility was checked by plating in a semi-solid medium of 0.65 per cent Bacto agar in tryptose-phosphate broth incubated at room-temperature. Highly motile colonies were selected for maintenance of the strain. Colonial

TABLE 1
Preparation of antigens

KILLING AGENT	TREATMENT
None (live)	—
Minimal heat	60 C, 15 minutes
Formalin	2%
Chloramine-T	0.1%
Merthiolate	0.1%
Phenol	2% for immunizing* 1% for adsorbing*

* Suspended in M/15 phosphate buffer.

morphology was controlled by plating on tryptose-phosphate agar and selecting colonies showing no evidence of roughness after seventy-two hours' incubation at 37 C. All of our strains exhibit more or less variability with respect to motility and roughness and even morphologically smooth strains react positively in the trypaflavine test (cf. Paterson).

Cultures to be used as antigens were grown in suitable volumes of tryptose-phosphate broth for 18–24 hours at room-temperature. Before processing samples were plated in semi-solid and on regular tryptose-phosphate agars as controls on motility and smoothness. Suspensions for treatment were prepared by centrifugation and resuspension to a density corresponding to a corrected Gates reading of 0.1 cm in physiological salt solution.

Concentrations of killing agents were selected such that they would be within the tolerable dose for rabbits, and the stock suspensions were maintained in these concentrations. Live antigens, used as controls, were kept in the cold room. The preparations used and their concentrations are given in table 1. The phenolized antigens frequently agglutinated spontaneously in the concentrations

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of phenol used. This was overcome by suspending in physiological salt solution buffered at pH 7.2 with M/15 phosphate mixture.

At times antigens, with the exception of formolized, became inagglutinable although retaining their specific capacity to adsorb antibody. Freshly prepared antigens were therefore used for testing for the removal of homologous antibody and agglutinative tests were carried out with stable formolized antigens.

For immunizing, except with live antigens, the stock suspensions were diluted to a corrected Gates reading of 1.45 cm with the proper concentration of killing agent in physiological salt solution. Rabbits were injected intravenously with graded doses from 0.1 ml to 1.0 ml on five successive days and the series repeated after a rest period of one week. If trial bleedings five to seven days after the last injection were unsatisfactory, additional courses were given. In the case of the live antigens the standard suspension was greatly diluted for the first course by adding one loopful to 10 ml of physiological salt solution. The volumes injected were as above. After the first course the standard suspension was used. At least two animals were immunized with each type of antigen, and in the absence of discrepancies additional animals were not treated.

The strains of *Listerella monocytogenes* represented all of the flagellar factors proposed by Paterson. Strain H-10-H (Gibson (5)), having flagellar factors BD, and strain B-8-H (Jungherr (6) 12159) with factors ABC were used in immunization. Strain H-4-H (Julianelle (7) PT) with the structure AB was also used in adsorbing and testing.

For the initial adsorptions a sufficient quantity of serum diluted 1:2.5 was added to an equal quantity of the stock suspension of the appropriate strain. In subsequent adsorptions the supernate was transferred to the sediment from a similar amount of stock suspension and the sediment resuspended. Adsorptions were for one hour at room-temperature. Successive adsorptions were carried out with each serum until no reaction was obtained with the adsorbing antigen.

Agglutinative tests were made in accordance with the usual technic of serial dilutions, the final dilution in the first tube of the series being 1:10. Test antigens consisted of formolized suspensions of the appropriate strain diluted to a corrected Gates reading of 1.45 cm. Tests were incubated for two hours at 50 C. Titers were recorded as the greatest dilution giving visible agglutination. All results were checked for reproducibility and, where apparent discrepancies occurred, the above procedures were repeated until the results were consistent.

Antibodies against individual factors were detected according to the schema of table 2. The results obtained by treatment of the antigens with minimal heat, formalin, chloramine-T, merthiolate, and phenol, as compared with live untreated antigens, are given in table 3. Titers of the unadsorbed serums are the reciprocals of the greatest dilution giving any agglutination of formolized suspensions of the homologous strains. The titers of factor-specific antibody are the reciprocals of the greatest dilution of appropriately treated serums giving any agglutination of formolized suspensions of the test organisms as indicated in table 2. The antigens used for adsorbing were treated with the appropriate

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killing agent. Essentially the same effects were produced by adsorption with live or formolized antigens.

The results indicate that there is little effect of the killing agent on the over-all response as evidenced by the unadsorbed titers, or on the "B" and "D" factors. On the other hand, there has been an obvious diminution in the activity of the "A" component in those antigens treated with chloramine-T, merthiolate, and

TABLE 2

Determination of individual factors

SERUM VS.	FACTOR	ADSORBED WITH	TEST WITH
H-10-H (BD)	B	—	H-4-H (AB)
	D	H-4-H (AB)	H-10-H (BD)
B-8-H (ABC)	A	H-10-H (BD)	H-4-H (AB)
	B	—	H-10-H (BD)
	C	H-4-H (AB)	B-8-H (ABC)

TABLE 3

Effect of various killing agents on specific flagellar factors

STRAIN	IMMUNIZING ANTIGEN KILLED BY	FLAGELLAR FACTORS								
			A		B		C		D	
		Titer of unadsorbed serum	Titer	Titer of unads/titer for A	Titer	Titer of unads/titer for B	Titer	Titer of unads/titer for C	Titer	Titer of unads/titer for D
H-10-H (BD)	(live)	20,480	—	—	10,240	2	—	—	10,240	2
	heat	2,560	—	—	1,280	2	—	—	1,280	2
	formalin	40,960	—	—	5,120	8	—	—	5,120*	8
	chloramine-T	5,120	—	—	1,280	4	—	—	640*	8
	merthiolate	10,240	—	—	5,120	2	—	—	320	32
	phenol	5,120	—	—	1,280	4	—	—	640*	8
B-8-H (ABC)	(live)	10,240	320	32	10,240	1	640	16	—	—
	heat	5,120	160	32	2,560	2	320	16	—	—
	formalin	40,960	1,280*	32	40,960	1	640*	64	—	—
	chloramine-T	20,480	40*	1,024	5,120	4	640	64	—	—
	merthiolate	20,480	20*	2,048	10,240	2	40*	1,024	—	—
	phenol	40,960	10*	4,096	20,480	2	1,280	32	—	—

* Titer slightly higher when adsorbed with live antigen.

phenol, where the total antibody content of the serum is from 1000 to 4000 times that of the factor-specific antibody, as opposed to the results obtained with the live and heat- or formalin-killed immunizing antigens, where the ratio is only thirty-two. This is in distinct contrast to the results obtained in testing for factor-specific antibodies against "B", "C", and "D", where the ratios are relatively uniform throughout. There appears to be an additional effect of merthiolate on the "C" component, but it is felt that this should be subjected to further investigation.

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The adsorptive capacity of the antigens subjected to different treatments varied considerably. In some instances the number of adsorptions required to remove the homologous antibody was the same for live and treated antigens, while with serums against other antigens as many as twenty-four successive adsorptions with the live antigen were required to produce the same effect as six to eight adsorptions with the treated antigen. Sufficient evidence is not at hand to permit interpretation of this phenomenon. These differences in adsorptive capacity were not reflected in the final results.

DISCUSSION

These results are admittedly preliminary in nature. No pretense can be made that the killing agent or preservative is necessarily the only factor which may alter profoundly the factor-specific nature of the antigen mosaic. Indeed we have presumptive evidence that in *Listerella* so elementary a difference as cultivation on a solid medium as opposed to broth of the same nutritive composition may produce marked qualitative differences in the antigenicity of the flagellar components.

There are, however, indications that those killing agents containing aromatic rings produce similar alterations in the responsiveness of individual factors even though this may not be reflected in the over-all response. These differences apparently did not appear in the results obtained by Ungar and his associates, but it is possibly significant that they used as a preservative phenol in sufficiently high concentration to have had some effect.

That the results obtained are not a reflection of degraded *in vitro* activity of the antigens involved is indicated by three different observations. First, these antigens acting *in toto* are fully as effective as live antigens or those killed by formalin or heat; second, testing for specific factors by adsorption with live antigens produced essentially the same results as with treated antigens; and third, the adsorptive capacity of the treated antigens was, in some instances, even greater than that of the live antigens.

It would be unwise to extend by analogy the results obtained with *Listerella monocytogenes* to other groups of bacteria. The organisms of this group are extremely variable and difficult to stabilize with respect to all of their morphological characteristics and it is possible that further investigation will reveal similar variability in their serological properties. It may well be that such sensitivity is reflected in greater susceptibility to change in antigenic factors than would be encountered in more stable groups of bacteria. On the other hand, the results obtained suggest the desirability of specifying minutely the conditions of cultivation and the method of killing and preservation of suspensions to be used in conducting antigenic analyses.

SUMMARY

1. The use of minimal heat, formalin, chloramine-T, merthiolate, or phenol as killing agents does not alter significantly the over-all capacity of *Listerella monocytogenes* to elicit the production of antibodies.

2. Flagellar factors B and D are not affected by any of the agents tested.

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3. Flagellar factor A is not affected by minimal heat or formalin but is degraded by chloramine-T, merthiolate, and phenol.
4. Flagellar factor C may be influenced by merthiolate.

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