STUDIES ON THE FLEXNER GROUP OF DYSENTERY BACILLI

V. A Quantitative Study of the Serological Cross-Reactions*

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The microorganisms which comprise the *Shigella paradysenteriae* group can be classified serologically into a number of types. Some of these types exhibit well defined specificities while others cross-react broadly. These observations led Andrewes and Inman (1) to propose that each type of Flexner dysentery bacillus contains a multiplicity of antigens. In their opinion the predominant antigen confers type specificity upon the microorganisms while the lesser antigens account for their serological cross-reactivities. Boyd (2) has presented an alternative explanation. He has suggested that each type of dysentery bacillus contains a single type-specific antigen and, in addition, an antigen common to the group. It is the latter which, in his opinion, accounts for the serological crossing of the various Flexner bacilli. The experiments reported in the preceding paper (3) are not entirely in agreement with this hypothesis, for it has been shown that the highly purified somatic antigens of the Flexner bacilli, as well as the polysaccharide haptens derived from them, exhibit all of the cross-reactions of the intact microorganisms themselves. The somatic antigens of several types of *Shigella paradysenteriae* have been obtained from the bacterial cells by different procedures, and the products of extraction have been subjected to a variety of chemical fractionations. When the immunologically active material has been finally separated from extraneous cellular substances, the end products, the somatic antigens, have shown remarkably constant analytical properties regardless of the method chosen for purification. In addition, the preparations studied have all exhibited electrophoretic homogeneity, a fact which suggests that the somatic antigens are indeed pure chemical substances.

It has recently been found, however, that the electrophoretic mobilities of the antigens derived from several different types of *Shigella paradysenteriae* are essentially the same.[1] Because of this fact, these substances might therefore still be considered as mixtures.

In order to test further the hypothesis that the purified antigens of Flexner

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[1] These observations have been made since the publication of our previous report (4).

dysentery bacilli are in reality single chemical substances, a quantitative study of their serological properties has been made. That these substances must be considered as single chemical entities, and not as mixtures of immunologically active components, will be seen from the following account.

TABLE I

The Cross-Reaction of the Somatic Antigens of Types I, III, and I-III Shigella paradysenteriae in Type I Antiserum

Antigen added	Antibody N precipitated*	Excess antigen in supernate (total)	Antibodies in supernate reactive with antigens of types		
			I	III	I-III
mg.	*mg.*	*μg.*	*per cent of original*	*per cent of original*	*per cent of original*
Type I antiserum *vs.* Type I antigen					
0.30	0.43	0	20	0	20
0.50	0.48	0	10	0	5
0.75	0.47	66	8	0	0
1.00	0.48	150	0	0	0
1.50	0.48	220	0	0	0
2.00	0.49	300	0	0	0
Type I antiserum *vs.* Type III antigen‡					
0.25	0.020	120	95	25	95
0.50	0.023	300	90	15	90
1.00	0.015	750	90	0	90
2.00	0.000	1500	85	0	90
Type I antiserum *vs.* Type I-III antigen					
0.25	0.31	10	35	60	30
0.50	0.42	54	12	50	10
1.00	0.42	200	9	50	5
1.50	0.43	330	7	50	0
2.00	0.40	600	7	50	0

* Corrected for the blank and the nitrogen in the precipitated antigen.

‡ Because of low values obtained 2 ml. of antiserum were used for each determination. The figures shown are all corrected for 1 ml. to aid in the comparison.

EXPERIMENTAL

Materials and Methods.—The specific somatic antigens of Types I, III, and I–III *Shigella paradysenteriae* were prepared from the respective microorganisms as previously described (4). Antisera were prepared by subjecting rabbits to a prolonged course of immunization with freshly grown, formol-killed dysentery bacilli of these three types. The quantitative precipitin nitrogen determinations were performed by the method of Heidelberger and Kendall (5).

The Reactions between the Somatic Antigens of Types I, III, and I-III Shigella paradysenteriae and Their Antisera.

The reaction between the somatic antigen Type I *Shigella paradysenteriae* and the homologous antibacterial serum was studied quantitatively by the procedure referred to. Similarly, the quantitative precipitation titration of two closely related heterologous antigens, Types III and I–III, was also ascertained. Antibody nitrogen determinations were performed in duplicate and the supernatant solutions from the duplicate tubes were pooled. They were tested quantitatively (6) both for excess antigen and for an excess of homologous and heterologous antibodies. Table I records the results of the quantitative determinations and Fig. 1 summarizes these same results in graphic form.

Types III and I–III antisera were likewise tested quantitatively for precipitative antibodies with the same three somatic antigens. The results of these studies are given in Tables II and III and in Figs. 2 and 3.

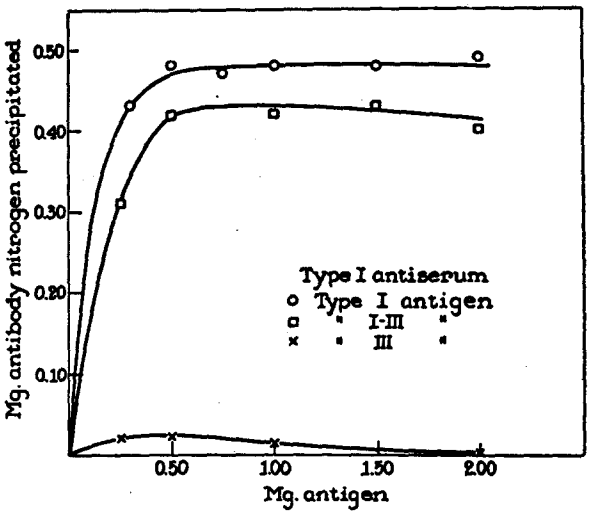

FIG. 1. The cross-reactions of the somatic antigens of Types I, III, and I–III *Shigella paradysenteriae* in Type I antiserum.

From the results presented in Tables I to III several general statements concerning the cross-reactions can be made. First, the somatic antigen is capable of removing all of the cross-reacting antibody from homologous antiserum, whereas the heterologous antigens, even in great excess, do not remove all of the homologous antibody. Far greater excesses than those reported here have been used and in no instance was it possible to exhaust the homologous antibody by means of the heterologous cross-reacting antigens. It can also be seen that the antibody nitrogen precipitated by heterologous antigen reaches a plateau which remains broad and shows no tendency to rise. These facts provide strong evidence that the somatic antigen of any single type of the Flexner bacillus is but a single substance and not a mixture of antigens of several types. If the latter were true it would be expected that a sufficiently great excess of heterologous antigen should contain enough homologous antigen to precipitate the antibody entirely.

The equivalence point in each of the three homologous antigen-antibody reactions is sharply defined. In view of the fact that the sera were obtained by prolonged immunization with intact microorganisms, secondary antigens, if they exist, have been given ample opportunity to evoke antibodies, yet no evidence for their presence has been obtained. It would appear, therefore, that the test antigens used are free of other antigenic substances.

TABLE II

The Cross-Reactions of the Somatic Antigens of Types I, III, and I-III Shigella paradysenteriae in Type III Antiserum

Antigen added	Antibody N precipitated*	Excess antigen in supernate (total)	Antibody in supernate reactive with antigens of types		
			III	I	I-III
mg.	*mg.*	*μg.*	*per cent of original*	*per cent of original*	*per cent of original*
Type III antiserum vs. Type III antigen					
0.25	0.33	0	50	50	50
0.50	0.46	18	6	10	10
1.00	0.50	65	3	3	0
2.00	0.51	190	0	0	0
Type III antiserum vs. Type I antigen‡					
0.25	0.04	30	95	50	65
0.50	0.07	108	90	15	55
1.00	0.09	400	90	0	55
2.00	0.05	1500	90	0	55
Type III antiserum vs. TypeI-III antigen					
0.25	0.14	20	70	40	40
0.50	0.21	84	60	12	12
1.00	0.23	300	60	0	0
2.00	0.21	1290	50	0	0

* Corrected for the blank and the nitrogen in the precipitated antigen.

‡ 1½ ml. serum were used for each determination but results shown are calculated for 1 ml. in order to aid in the comparison.

Another fact which is evident from the experimental results is that a single heterologous antigen does not exhaust a serum of the antibody reactive with a second heterologous antigen. For example, Type III specific antigen does not remove from Type I antiserum all of the antibody reactive with the Type I–III heterologous antigen. One apparent exception is seen in Table II where Type I–III antigen removes from Type III antiserum all the Type I antibody. in this instance, however, the amount of Type I antibody initially present in the Type III antiserum was so small that when a portion of the antibody was

removed, the amount remaining could not be detected by the analytical method used.

The data presented in Tables I to III also reveal that the heterologous antigens always remove some of the antibody reactive with the other heterolo-

TABLE III

The Cross-Reactions of the Somatic Antigens of Types I, III and I-III Shigella paradysenteriae in Type I-III Antiserum

Antigen added	Antibody N precipitated*	Excess antigen in supernate (total)	Antibody in supernate reactive with antigens of types		
			I-III	I	III
mg.	*mg.*	*μg.*	*per cent of original*	*per cent of original*	*per cent of original*
Type I-III antiserum *vs.* Type I-III antigen					
0.10	0.27	0	35	35	30
0.20	0.34	0	13	15	15
0.30	0.42	5	10	10	10
0.50	0.43	23	4	0	0
0.75	0.43	50	0	0	0
1.00	0.42	70	0	0	0
Type I-III antiserum *vs.* Type I antigen					
0.25	0.20	0	50	30	80
0.50	0.27	48	35	10	80
1.00	0.31	120	30	2	80
1.50	0.30	180	30	0	80
2.00	0.32	360	30	0	80
Type I-III antiserum *vs.* Type III antigen‡					
0.25	0.10	105	80	40	10
0.50	0.14	240	60	40	1
0.75	0.14	420	60	40	0
1.00	0.14	630	60	40	0
1.50	0.12	1000	60	40	0
2.00	0.10	1500	60	40	0

* Corrected for the blank and the nitrogen in the precipitated antigen.

‡ 1½ ml. of serum were used for each determination but results shown are calculated for 1 ml. in order to aid in the comparison.

gous antigen. In addition, the amount of antibody precipitated by a heterologous antigen is equivalent to the decrease in homologous antibody. Thus, Type I–III antiserum contains 0.45 mg. of antibody nitrogen precipitable with the homologous antigen. The Type I antigen precipitates from this immune serum 0.31 mg. of antibody nitrogen. This leaves (0.43 − 0.31 = 0.12) 0.12 mg. of antibody nitrogen remaining in solution. A direct quantitative

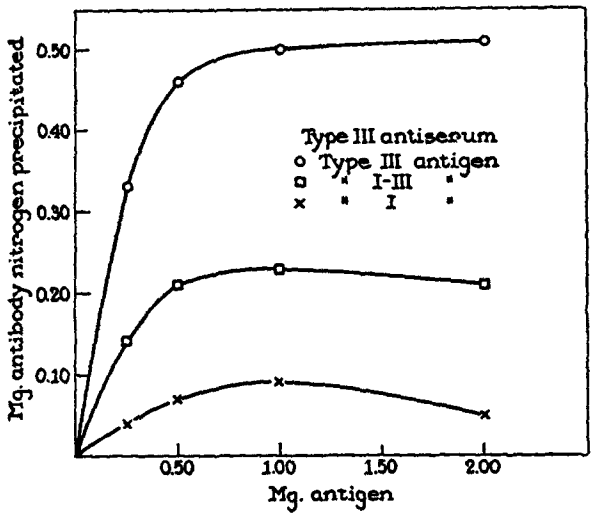

FIG. 2. The cross-reactions of the somatic antigens of Types I, III, and I–III *Shigella paradysenteriae* in Type III antiserum.

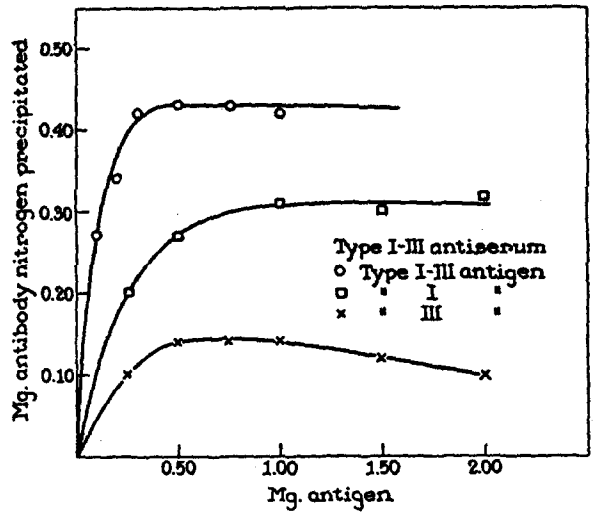

FIG. 3. The cross-reactions of the somatic antigens of Types I, III, and I–III *Shigella paradysenteriae* in Type I–III antiserum.

test of the supernate shows that 30 per cent of the original homologous antibody is left. This value agrees well with the calculated amount, $\frac{0.12}{0.43} \times 100 = 28$ per cent. It can therefore be concluded that the Type I–III antiserum con-

tains several kinds of antibodies, all of which are precipitable by the homologous antigen. Some of the antibody molecules react only with the homologous Type I–III antigen. Others are reactive with both the homologous antigen and the Type I antigen as well. Another group of antibody molecules reacts with the homologous and with the Type III antigens; still others are reactive with all three antigens. Further evidence for this hypothesis was obtained by eluting (7) the antibody obtained from the precipitate of Type I–III antiserum and Type III antigen. The recovered antibody was found to contain agglutinins in high titre for Type I–III and Type III bacilli and demonstrable agglutinins for Type I microorganisms as well.

While it is apparent that the three antigens, Types I, III, and I–III are interrelated serologically, it is to be noted that the Type I–III antigen is more

TABLE IV

The Reaction between Type I-III Antiserum and a Mixture of Type I and Type III Somatic Antigens

Antigen added		Antibody nitrogen precipitated*	Excess antigen in supernates		Excess antibody in supernatant		
Type I	Type III		Type I	Type III	Type I	Type III	Type I-III
mg.	*mg.*	*mg.*	*μg.*	*μg.*	*per cent of original*	*per cent of original*	*per cent of original*
1.00	1.00	0.33	150	660	tr	0	30
2.00	2.00	0.32	500	1000	0	0	30
3.00	2.00	0.33	1000	1000	0	0	30

* Corrected for the blank and for the nitrogen contained in the precipitated antigen.

closely allied to the Type I than to the Type III antigen. The Type I–III antigen, on the other hand, is more closely related to the remaining two heterologous antigens than are the latter to each other.

The Reaction between Type I–III Antiserum and a Mixture of Types I and III Specific Antigens.

In order to demonstrate the presence of antibodies in Type I–III antiserum which are specifically reactive only with the homologous antigen the following experiment was performed. Type I–III antiserum was precipitated with a mixture of Types I and III antigen in varying quantities and proportions. The antibody nitrogen precipitated was determined in the usual manner. The supernatant liquids were in each instance tested quantitatively for excess antibody with the homologous and heterologous antigens. In order to determine the excess of the two heterologous antigens in the supernates, it was necessary first to prepare monospecific antisera. Type I monospecific antiserum was obtained by absorbing the original serum with an excess of Type III antigen. Similarly monospecific Type III antiserum was prepared by absorption with an excess of Type I antigen.

The results of the quantitative studies are presented in Table IV where it can be seen that the antibody nitrogen precipitated by the mixture of Types I and

III antigens is 0.33 mg. The Type I antigen alone precipitated 0.31 mg. of antibody nitrogen from this same antiserum and the Type III antigen alone precipitated 0.14 mg. (Table III). The mixture of Types I and III antigens has precipitated but slightly more antibody than has the Type I antigen alone. Since the homologous antigen precipitates 0.43 mg. of antibody nitrogen it is evident that the mixture of heterologous antigens removes but 70 per cent of the total precipitable immune bodies. This finding offers additional support to the contention that TypeI–III antiserum contains antibody molecules reactive solely and specifically with the homologous Type I–III antigen, and that a good portion of the cross-reactive antibody molecules are reactive with both Types I and III antigens.

An attempt was also made to exhaust the homologous antibody from a Type I antiserum by the addition of varying amounts of a mixture of Type I–III and Type III antigens. Although the protocols are not included, the results were similar to those reported in detail above. In no case was it possible to remove all of the homologous antibody. The cross-absorption of Type I as well as of Type I–III antiserum is never complete, and the cross-reactions of these two types appear to be non-reciprocal. Thus it can be stated that although the Type I and Type I–III somatic antigens are indeed closely related serologically, they cannot be considered identical.

The Partial Precipitation of Type I–III Somatic Antigen.—Because Type I–III microorganisms are so closely related serologically to those of Types I and III, it was suggested by Andrewes and Inman (1) that the Type I–III bacillus contains for the most part a mixture of Type I and Type III antigens. If this is true, it should be possible to separate the components by selective precipitation with monospecific antisera.

Separate samples of the Type I–III somatic antigen were partially precipitated with monospecific Type I and Type III antisera. In each instance the residual, unprecipitated antigen remaining in the supernate was tested quantitatively for its reactivity in the two monospecific antisera. The results are compared with those obtained from a similar control experiment in which the Type I–III antigen was partially precipitated by its homologous antiserum.

The monospecific antisera were carefully prepared in the following manner. Type I antiserum was absorbed with a slight excess of Type III antigen in such a manner that not more than 5 μg. per ml. of the antigen remained in the supernate. Similarly a monospecific Type III antiserum was prepared by absorption with Type I antigen. The partial precipitation of the Type I–III somatic antigen was performed as follows: a solution of the antigen was prepared by dissolving 750 μg. per ml. of M/15 phosphate buffer at pH 7.6. Two ml. aliquots of this solution were mixed respectively with 1 ml. of the two monospecific antisera and a third aliquot was mixed with 1 ml. of the homologous Type I–III antiserum. In each instance the concentration of serum used was so chosen as to precipitate approximately two-thirds of the Type I–III antigen. The mixtures were incubated for 2 hours at 37°C. and placed at 4°C. for 3 days. The tubes were then centrifuged in the cold and the precipitates discarded. Serial dilutions of each of the three supernates were added to a constant volume of the monospecific Type I and III antisera. The turbidities developed were determined photometrically. By comparing these turbidities with those developed by known amounts of Types I and III

antigens in their respective antisera, it is possible to ascertain the amount of antigen in these supernates reactive with Types I and III antiserum. In each instance the ratio of the apparent amount of Type I reactive material to the amount of Type III reactive material was calculated. These values are shown in Table V.

If the antigen obtained from Type I–III microorganisms is a mixture of Type I and Type III antigens, partial preciptation of this mixture with monospecific Type I antiserum should decrease the ratio of Type I to Type III reactive material in the supernate. Likewise partial precipitation with the monospecific Type III antiserum should result in an increase of this ratio. As can be seen from Table V, however, there is no significant alteration of this ratio. It can be concluded therefore that the somatic antigen of Type I–III *Shigella paradysenteriae* is not a mixture of two immunologically active substances but that it is a single substance.

TABLE V

The Serological Activity of Type I-III Antigen after Partial Precipitation with Monospecific Antisera

Type I-III antigen tested after partial precipitation with	Ratio of serological activity Type I/Type III
Monospecific Type I antiserum	5.1/2.1
Monospecific Type III antiserum	5.0/2.1
Type I-III antiserum	5.0/2.0

DISCUSSION

Investigations on the chemical constitution of bacterial antigens have shed considerable light upon the factors which govern the serological cross-reactions encountered among microorganisms. It is known that some bacteria contain not only type-specific but group-specific antigens as well (8). In certain instances the common group antigens are responsible for the serological cross-reactions among members of a bacterial species, yet it has been demonstrated that similarities in the chemical structures of type-specific antigens can account for their serological crossing (9–11).

In the case of the microorganisms which comprise the Flexner group of dysentery bacilli we are faced with the problem of explaining their serological crossing. The phenomenon can be attributed either to the presence of a common group antigen as proposed by Boyd (2), to mixtures of serologically active components as suggested by Andrewes and Inman (1), or, as postulated in the present communication, to similarities in the chemical structure of the somatic antigen of each individual type. In explaining the serological cross-reactions among microorganisms of the *Salmonella* group, Meyer (12) has come to the conclusion that the last hypothesis is the most tenable.

Our chemical work has indicated that the purified somatic antigens of the Flexner bacilli are single, individual substances which cannot be further fractionated by physical or chemical means. Yet the properties of these substances are so closely allied one cannot disregard the possibility that they might still be mixtures. The results of our present investigations have provided additional evidence, however, for the chemical individuality of the somatic antigens of three representative types of the Flexner group, Types I, III, and I–III.

This evidence has been obtained from a detailed study of the serological behavior of the highly purified antigens in homologous and heterologous antisera. The three homologous antigen-antibody reactions exhibit sharply defined equivalence points. In systems composed of mixtures of two or more antigens and their respective antibodies the occurrence of such a sharp equivalence point is possible, but highly unlikely. From the experimental data presented it has been shown that in the three sera studied the complete precipitation of the antibody reactive with one or both heterologous antigens failed to remove all of the homologous antibody. If the heterologous antigen were a mixture of immunologically active components one of which is homologous to the serum, it should have been possible to absorb the homologous immune body provided a sufficient excess of the antigen had been added. That this was not the case, however, is evident from the experiments recorded.

It was shown furthermore, that one heterologous antigen, even in excess, failed to exhaust an antiserum of the immune bodies reactive with a second heterologous antigen. It is this fact which excludes the possibility of a common group antigen being present in our preparations of the somatic antigens of *Shigella paradysenteriae* (Flexner).

Finally, it may be pointed out that all attempts to fractionate the Type I–III antigen by partial precipitation with monospecific antisera were unsuccessful. One must conclude therefore that the cross-reactive Type I–III antigen is in reality a single chemical substance, and not a mixture of Type I and Type III antigen. Similar experiments have been performed using the somatic antigens of other types of the Flexner group. Although protocols have not been presented, the results obtained were in all respects similar to those reported in detail in the present communication. We feel confident, therefore, that all experimental evidence points toward the fact that the purified somatic antigens of the Flexner group of dysentery bacilli are single chemical entities endowed with multiple serological reactivities. This concept is by no means at variance with modern theories regarding the cross-reactions of antigen-antibody systems. In his critical review Landsteiner (13) has emphasized the fact that artificially prepared antigens containing groupings of known chemical constitution may give rise to immune bodies which cross-react with antigens having similar though not identical groupings. It is the similarity in chemical constitution of the carbohydrate components of the somatic antigens of Flexner bacilli which, in our opinion, determines their serological crossing.

SUMMARY

A quantitative study of the serological cross-reactions of the somatic antigens of Types I, III, and I-III *Shigella paradysenteriae* has been made.

From the results obtained it has been concluded that the somatic antigens of *Shigella paradysenteriae* (Flexner) Types I, III, and I–III are single chemical substances. The specificity and serological cross-reactions exhibited by these antigens are dependent upon similarities in their chemical constitutions.

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