STUDIES ON THE TRANSFER OF LYMPH NODE CELLS

VII. Transfer of Cells Incubated in Vitro with Filtrates of Trypsin-Treated Suspensions of Shigella Paradysenteriae*

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In an earlier study of this series it was found that if cells of the popliteal lymph nodes of rabbits not previously injected with dysentery bacilli were incubated *in vitro* with suspensions of these organisms, and were then washed and transferred to x-irradiated recipient rabbits, agglutinins to these organisms appeared in the sera of the recipients after a few days (1). Similar results were later obtained when the lymph node cells were incubated *in vitro* with rabbit serum which had been treated by incubation with dysentery bacilli and subsequent removal of these organisms by centrifugation and Seitz filtration (2). From the latter study it appeared that soluble antigenic material derived from dysentery bacilli was present in serum so treated, and that this material could serve as an effective antigen when incubated *in vitro* with lymph node cells. The possibility was then investigated that enzymatic digestion of whole dysentery bacilli might yield such antigenic material in a form preferable to that of the *Shigella*-treated serum for both qualitative and quantitative study. Because of the proteolytic nature of trypsin and its availability in crystalline form, this enzyme was employed at the outset.

Preparation of soluble antigenic material from *Shigella paradysenteriae* by tryptic digestion had been reported by Goebel *et al.* in 1945 (3, 4). This material was obtained on 2 to 3 days of digestion of glycol-extracted or unextracted *Shigella*, and was considered to be a degradation product of the antigenic lipoprotein-carbohydrate complex extractable from these organisms. Both this complex and the "tryptic" antigen produced precipitates with antisera to *Shigella*, and gave rise to precipitating antibodies on injection into rabbits.

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Materials and Methods

Animals.—The rabbits used as donors of the lymph node cells were adult animals of approximately 2 kg. These donor rabbits either were uninjected or had received injections into the hind and fore foot pads of 0.2 ml. of packed sheep erythrocytes 4 days prior to sacrifice in order to increase the yield of cells per node. Recipient rabbits weighed approximately 1 kg.

Preparation of Cell Suspensions.—The popliteal and usually the axillary lymph nodes of the donor rabbits were excised. Cells were liberated by teasing the nodes into Tyrode's solution containing 0.13 per cent gelatin, in which Na_2HPO_4, 0.01 M, was substituted for the $NaHCO_3$. Phenol red was incorporated in the Tyrode's solution. As teasing progressed the pH of the suspending medium generally remained above 7.0. On the rare occasion when the pH fell below this level the addition of 1 to 2 drops of 0.75 M NaOH was sufficient to restore the pH to neutrality. The number of available lymph node cells was estimated on the basis of the volume of the suspension and the turbidity as determined in a Klett colorimeter. After centrifugation for 5 minutes at 1400 R.P.M., the cell sediment was taken up to a volume which would contain approximately 250×10^6 cells per ml. The cell suspension was incubated with an equal volume of the solution of antigenic material for 30 minutes at 37°C. in slowly rotating tubes. Thereafter the suspension was centrifuged for 5 minutes at 1400 R.P.M., and the cell sediment was washed two times in 40 volumes of Tyrode-gelatin solution and then in 20 volumes of the Tyrode's solution containing 7½ per cent normal rabbit serum. The final cell sediment was suspended in 5 additional volumes of Tyrode rabbit serum solution.

Portions of the 1:6 cell suspension were injected untreated into recipients which had been x-irradiated 24 hours earlier, and portions were heated at 60°C. for 60 minutes prior to transfer. Samples were also taken for determinations of the number of cells present and of the percentage viability of the cells, as determined by their failure to take up trypan blue.

Preparation of the Soluble Antigenic Material.—*Shigella paradysenteriae* cultures in the logarithmic phase of growth were seeded on tryptose agar in Blake bottles or into trypticase soy broth (Baltimore Biological Laboratories). After 6 hours of cultivation at 37°C. the organisms were collected by washing the culture off the agar with sterile saline solution, or, in the case of the broth culture, by centrifugation in the Sharples centrifuge at 40,000 R.P.M. The bacterial sediment was washed twice in saline, suspended for 3 hours in 70 per cent ethyl alcohol, washed twice, and maintained as a 10 per cent suspension in Tyrode's solution for 48 hours at 4°C. The bacterial cell mass was then collected by centrifugation, resuspended at 10 per cent in Tyrode's solution, pH 8, which contained crystalline trypsin and merthiolate at final concentrations of 1 mg. per ml. and 0.1 mg. per ml., respectively. After 20 hours of incubation at 37°C. the organisms were removed from the suspension by centrifugation followed by Seitz filtration. Control preparations were made of each suspension of organisms, in which the trypsin was omitted from the suspension incubated at 37°C.

Antiserum.—Serum was obtained from a group of adult rabbits which had been injected intravenously with suspensions of alcohol-treated *Shigella paradysenteriae* and bled 6 days later. The agglutinin titer of this serum was 6000, by the technic described in the following paragraph. The serum also gave complement-fixation and precipitin reactions with the soluble antigenic materials prepared from *Shigella paradysenteriae* as described above.

Serologic Tests. Agglutination.—Serial two-fold dilutions of rabbit serum were made in volumes of 0.4 ml. To these was added 0.2 ml. of a 0.06 per cent suspension of alcohol-treated *Shigella paradysenteriae.* After shaking and 1 hour of incubation at 37°C., the tubes were stored at 4°C. for 48 hours, and then examined for evidence of agglutination, as described in detail previously (5).

Complement-Fixation.—Complement-fixation tests for relative concentrations of the soluble antigenic material in the filtrates of trypsin-treated or control suspensions of *Shigella* were performed by incubating serial twofold dilutions of such preparations, in volumes of 0.4 ml.,

with 0.1 ml. of complement (1.5 units) and 0.1 ml. of the antiserum described above at a concentration of 1:12. This concentration of serum had been determined previously as the optimal concentration in block titrations of dysentery-trypsin filtrates *versus* this serum. After incubation at 37°C. for 45 minutes to allow for the fixation of complement, each tube received 0.2 ml. of sensitized sheep erythrocytes (equal volumes of a 4 per cent suspension of the erythrocytes and of an appropriate dilution of a rabbit anti sheep-erythrocyte serum). After further incubation for 30 minutes at 37°C., and overnight at 4°C., the degree of hemolysis was read in each tube, the end point being taken at the highest dilution of the antigenic filtrate which yielded no hemolysis in this test.

Precipitin Tests.—Precipitin tests were carried out by incubating dilutions of the filtrates, in volumes of 0.2 ml. with 0.1 ml. of the antiserum described above. After 48 hours of incubation at 37°C. and centrifugation for 10 minutes at 2000 R.P.M. the tubes were shaken and the relative volume of any precipitate present was estimated within the following grades: 0, trace, 1, 2, 3, 4.

Inhibition of Agglutination of Dysentery Organisms.—Another procedure used for relative estimation of the concentration of soluble antigenic material in various preparations was the inhibition of agglutination. In this procedure serial 2-fold dilutions of the filtrate in volumes of 0.4 ml. were incubated for 30 minutes at 37°C. with 0.2 ml. of a 1:2000 dilution of the antiserum to *Shigella* described above. After this incubation 0.2 ml. of a suspension of *Shigella* was added to each tube at the concentration used in the agglutination test, and further incubation was carried out for 1 hour at 37°C. and then 48 hours at 4°C. The presence or absence of agglutination was then noted as in the case of the agglutination test.

Irradiation of Recipient Rabbits.—The animals were exposed, on the day before cell transfer to 425 r of deep Roentgen rays, with the following factors: 200 kv., 20 ma., 67.5 cm. distance to the bottom of the container, yielding 18 r per minute in air. Filtration was by 1 mm. aluminum + 0.5 mm. copper.

EXPERIMENTAL

1. Serologic Evidence for the Presence of Soluble Antigenic Material in Filtrates of Trypsin-Treated Suspensions of Dysentery Organisms.—After a number of preliminary experiments, the great majority of suspensions of dysentery organisms involved in this study were treated with trypsin by the technic described above; *i.e.*, with final concentrations of organisms and crystalline trypsin of 10 per cent and 0.1 per cent, respectively, and incubation at 37°C. for 20 hours before removal of the bacterial cells. The resulting filtrates, on being tested by complement-fixation *versus* an anti whole *Shigella* rabbit serum, gave titers ranging from 128 to 1024, of which typical examples are shown in Table I.

In the case of a number of these preparations tests by precipitation and by inhibition of agglutination were also carried out. These were positive, indicating the presence of antigenic material derived from *Shigella*. A linear relationship was observed between the estimates of the concentration of antigenic material in the respective preparations by each of these tests and those of the titers by complement fixation. The precipitin titers were between one-quarter and one-sixth of the corresponding complement-fixation titers, and the titers by inhibition of agglutination were between two and three times as high as those by complement fixation.

Filtrates of trypsin-treated suspensions of bacilli were compared in complement-fixation tests with control filtrates of *Shigella* suspended in Tyrode's solution. The results, as shown in Table I, indicated that complement-fixing activity was present in control preparations but usually in substantially lower concentration.

The effects of variations in the conditions of the incubation with trypsin were studied in terms of the concentration of the enzyme and the period of

TABLE I

Complement-Fixation Titers of Filtrates of Trypsin-Treated and Control Suspensions of Dysentery Organisms

Preparation No.	Trypsin-treated		Control (Tyrode)	Ratio of trypsin:tyrode
	Hrs. of digestion	Complement-fixation titer	Complement-fixation titer	
16	4	128	4	32
20	4	128	32	4
21	4	128	<8	>16
26 *a*	4	768	128	6
26 *b*	20	1024	192	5.3
27 *a*	4	256	<48	>5.3
27 *b*	20	1024	128	8
28	4	256	32	8
30 *a*	4	192	32	6
30 *b*	20	512	64	8
34	20	512	64	8
35 *a*	20	1024	48	5.3
35 *b*	20	768	96	8
35 *c*	20	1024	64	16
36	20	512	32	16
39	20	192	32	16
40 *a*	4	256	16	16
40 *b*	20	512	32	16
45	20	256	24	10.7
46	20	512	48	10.7

incubation. With decreasing concentrations of trypsin from 1 mg./ml. of bacterial suspension to 10^{-4} mg./ml., decreasing concentrations of complement-fixing material were found, over approximately an 8-fold range. On increasing the time of incubation from 20 minutes to 20 hours, a 30-fold increase was noted in the concentration of complement-fixing material in the filtrates. These findings are summarized in Table II.

2. Transfer of Lymph Node Cells Incubated with Filtrates of Trypsin-Treated and Control Suspensions of Dysentery Organisms.—The filtrates of trypsin-treated suspensions of bacilli were used as a source of antigenic material and

incubated *in vitro* with lymph node cells obtained from uninjected donor rabbits, or from donors injected with heterologous antigen. After incubation with filtrates in given dilutions the lymph node cells were washed three times and transferred to recipient rabbits which had been irradiated 24 hours earlier. Agglutinins to dysentery bacilli were detected in the sera of the recipient

TABLE II

Effect of Varying the Time of Incubation and the Concentration of Trypsin on the Complement-Fixation Titer of the Dysentery-Trypsin Filtrate

Various times of incubation									
DT preparation.....	20		21		27	30	32	41	
Trypsin, *mg. per ml.*.....	1	0	1	0	1	1	1	1	0
Time of incubation	Complement-fixation titer								
0								32	32
20 min.	32	12	32	12				64	
60 "	48	12	32	12				128	
2 hrs.								256	
4 "	128	24	128	12	256	192	384	256	32
8 "								384	
20 "					1024	512	1024		

Various Concentrations of Trypsin		
DT preparation.....	28	41
Hrs. of incubation.....	4	20
Concentration of trypsin	Complement-fixation titer	
mg./ml.		
0	—	32
0.0001	48	64
0.001	—	64
0.01	192	128
0.1	—	384
1.0	256	512

rabbits 3 or 4 days after the cell transfer, usually on the 4th day. The agglutinin titer rose and reached a maximum on the 6th to 8th day, after which it declined. Recipients of portions of the cell suspension which had been heated at 60°C. for 1 hour prior to transfer did not develop agglutinins to dysentery bacilli in the 1st week after transfer. In the early experiments with the filtrates of trypsin-treated bacilli two preparations were used extensively. Agglutinin titers of the sera of 58 recipients of cells which had been incubated

with one of these were tabulated and grouped by the level of the maximum titer attained. Within each such group geometric mean titers were calculated for given intervals after transfer. Fig. 1 A illustrates the types of antibody curves observed in these experiments, with an indication of the number of recipients included in each geometric mean. The other preparation had been used for incubation with lymph node cells in 6 experiments, representing a total of 25 recipients. In this case the titers found in the sera of recipients were grouped by experiment, for presentation in Fig. 1 B. The curves obtained in this part of Fig. 1 resemble those shown in Fig. 1 A. (The slight difference in the shape of the curves is due to the omission of a 5th day blood specimen in the experiments summarized in Fig. 1 B.)

In many experiments lymph node cells were incubated with filtrates of control suspensions of bacilli which had been incubated in Tyrode's solution without trypsin, as well as those treated with trypsin. The ranges of complement-fixation titers of the two types of preparations have been shown above. Agglutinins to *Shigella* were found in sera of recipients of lymph node cells incubated *in vitro* with filtrates of the control suspensions, but these were as a group substantially lower in titer than those found in recipients of cells incubated with filtrates of trypsin-treated suspensions. Table III shows the results of a number of such comparisons in both complement-fixation and cell transfer.

3. Titration of Filtrates of Trypsin-Treated Bacilli in Cell-Transfer Experiments.—Titration of the filtrates of trypsin-treated bacilli was undertaken in order to study the relation between relative concentrations of the active material used for incubation with lymph node cells and serum titers of recipients of such cells. These experiments were carried out with a number of filtrate preparations. In each case the preparation was diluted serially by 10-fold steps. Aliquots of the suspension of lymph node cells were incubated with each of the dilutions of filtrate, and after incubation and subsequent washing the cells were transferred to irradiated recipients. When possible a portion of the cell suspension which had been incubated with the highest concentration of antigen was heated and then transferred to normal recipients. This served as a sensitive measure of antigen present in the cell suspension in that the recipients were not irradiated and could actively form antibody. From a group of such titration experiments it could be seen that for most preparations of the filtrate there appeared to be a range of concentration within which the level of agglutinins which appeared subsequently in the sera of recipients was independent of that concentration. Thus, in the case of almost all the preparations tested the maximum agglutinin titers in recipient rabbits were within a fairly narrow range when the cells had been incubated with filtrates at concentrations of 4×10^{-3}, 10^{-3}, and 10^{-4}. The recipients' titers were usually lower when the filtrate was used at a concentration of 10^{-5}, and quite low when the filtrate was used at a concentration of 10^{-6}. Such titrations, or at least the comparisons of critical dilutions, were always performed

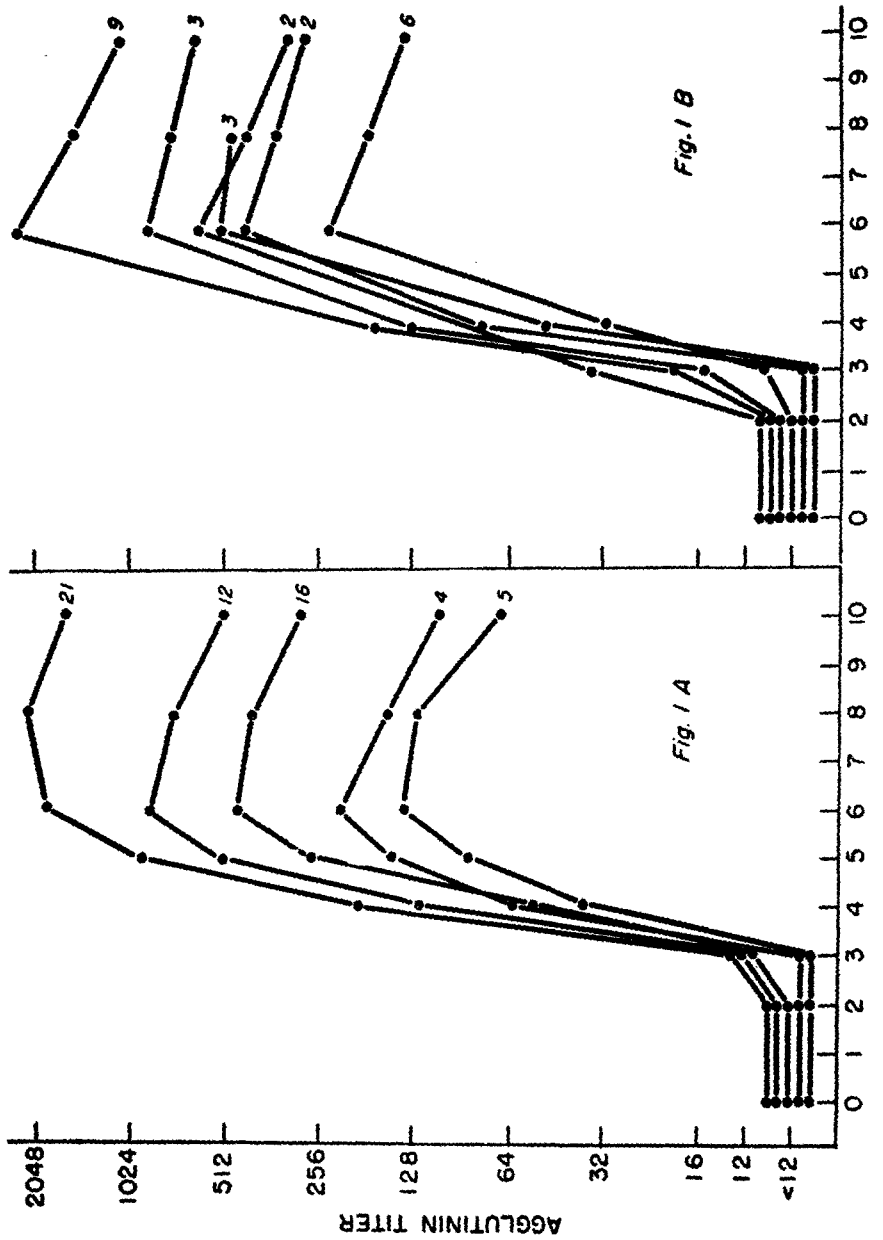

FIG. 1. Geometric mean agglutinin titers of recipients of lymph node cells incubated *in vitro* with filtrates of trypsin-treated suspensions of *Shigella paradysenteriae*. Fig. 1 A shows data obtained with one preparation; the curves represent geometric means of groups of similar titers obtained in different recipients (the number of these is indicated in the case of each curve). Fig. 1 B shows data obtained with another preparation; in this case geometric means were calculated from data in individual experiments.

within a given experiment, in order to minimize the effects of variations in the cell suspensions and other experimental conditions. Four filtrate preparations were tested extensively and the results obtained in the different experiments are illustrated in Fig. 2. In the case of each of these preparations the maximum agglutinin titer of each recipient is given for each dilution tested. Geometric means of the maximum titers at each concentration of filtrate are connected by the broken lines.

Preparations of such dialyzed filtrates yielded a dry weight of 1 mg./ml. or somewhat less. A preparation of such a dialyzed filtrate, which had been purified with respect to the active material to a point of apparent homogeneity on paper electrophoresis had a dry weight of approximately 0.5 mg./ml.

TABLE III

Geometric Means of Maximum Agglutinin Titers of Recipients of Lymph Node Cells Incubated with Filtrates of Trypsin-Treated and Control Suspensions of Dysentery Organisms

DT preparation No.	Trypsin			Tyrode		
	Complement-fixation titer	Cell transfer		Complement-fixation titer	Cell transfer	
		No. of recipients	Geometric mean peak titer		No. of recipients	Geometric mean peak titer
16	128	58	809	4	2	48
18	512	8	192	192	8	60
20	128	2	512	32	2	24
21	128	2	512	<8	2	12
26	1024	4	973	192	4	96
27	1024	25	784	128	2	112
46	512	6	426	48	2	40

Analyses of the latter yielded the following values: C, 42.82 per cent; H, 6.99 per cent; N, 5.95 per cent.

4. Effect of the Addition of Anti Whole Shigella Rabbit Serum during Incubation.—Attempts were made to determine what effect anti whole *Shigella* rabbit serum would have on the *in vitro* incubation of lymph node cells with the filtrate of trypsin-treated suspensions of dysentery bacilli. In the first experiment of this series, included in Table IV, a portion of the suspension of lymph node cells was incubated at 37°C. for 30 minutes with the filtrate, then washed and transferred to irradiated recipients in the usual manner (group 1). Another portion of the cell suspension was incubated with the filtrate for a given period of time and then antiserum in substantial excess was added. After further incubation of the mixture at 37°C. for 15 minutes, the cells were washed and transferred to irradiated recipients (group 2). A third portion of the cell suspension was incubated with antiserum for 15 minutes at 37°C. before addition of the antigenic material and further incuba-

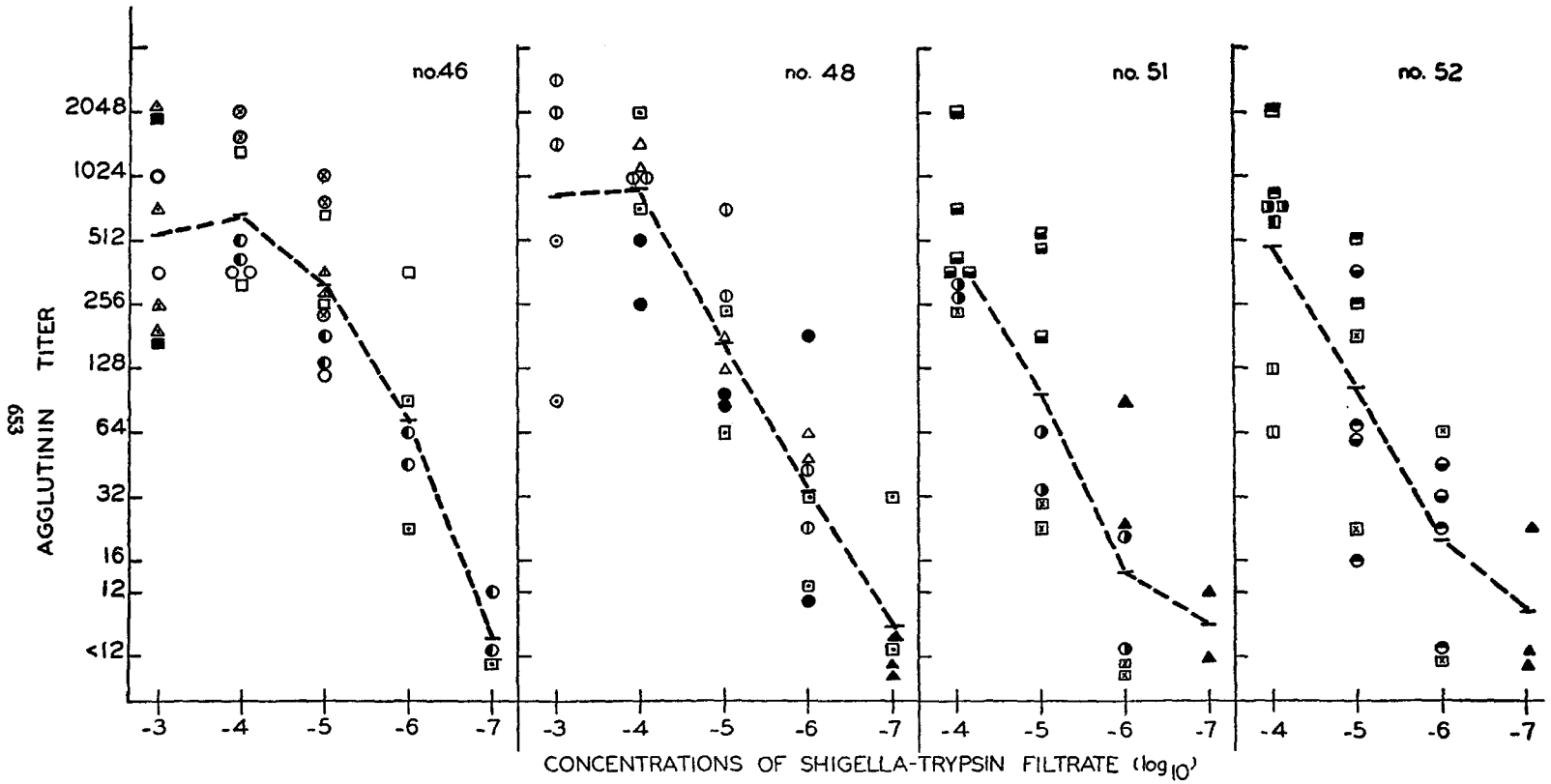

FIG. 2. Maximal agglutinin titers in sera of recipients of lymph node cells which had been incubated with various concentrations of 4 preparations of *Shigella* trypsin filtrate. The lines connect the geometric mean for each concentration of each filtrate. The various symbols indicate different experiments.

tion at this temperature (group 3). To a fourth portion of the cell suspension was added a mixture of filtrate and antiserum which had been incubated together for 15 minutes at 37°C. After further incubation the cell suspension was washed and transferred to irradiated recipients (group 4). When the maximum agglutinin titers of recipients in the different groups were compared it was found that whereas the usual incubation of cells with antigen before transfer yielded agglutinin titers such as those shown in the first column

TABLE IV

Effect of the Addition of Shigella paradysenteriae Antiserum at Various Times in Relation to the Incubation of Lymph Node Cells and Antigen

Experiment No.	Maximum agglutin titer of individual recipients								
	Group 1	Group 2						Group 3	Group 4
	Control: Tyrode's solution or normal rabbit serum	Lymph node cells incubated with filtrate; antiserum added at various times after beginning of the incubation (min.)						Antiserum added to cells before filtrate	Antiserum incubated with filtrate; mixture added to cells
		30	20	10	5	2	0.5-1		
421	1536	768						12	16
	2048	1024						24	64
									16
433	768	384	768	384					12
	768	1024	1536	768					16
439	1536			1536	1024	192	48		
	1536			1536	1536	768	96		
443	1536				768		32	96	64
	1536				768		192	32	<12
470					1536	384		16	<12
					192			24	12
									12
									48

(group 1), incubation of the filtrate with antiserum in excess before the addition of the cells caused a marked decrease in the recipients' agglutinin titer after the cell transfer (group 4). Similar results were obtained when the antiserum was added at the same concentration to the cells before the addition of the filtrate to the incubation mixture (group 3). If, however, cells and filtrate were incubated for the usual period of 30 minutes at 37°C., the subsequent addition of antiserum did not cause a reduction in the agglutinin titers of the recipients (group 2). (Experiment 421 in Table IV.) In the subsequent experiments normal rabbit serum was used in control groups, at the same

dilution as the immune serum. This was found to have no effect on the level of agglutinin titer which developed in the recipients. In further experiments the filtrate was added to lymph node cells and incubated together at 37°C. for various periods from 30 minutes to less than 1 minute, after which the same amount of antiserum was added and further incubation allowed. In this series of experiments it was found that adding the antiserum at 20, 10, or 5 minutes after the beginning of the incubation of cells and antigen appeared to have no effect on the level of agglutinins which developed in the recipients, in comparison with control material containing normal rabbit serum, as shown in group 2 of the table. When the serum was added 2 minutes after incubation of cells and filtrate there was a questionable reduction

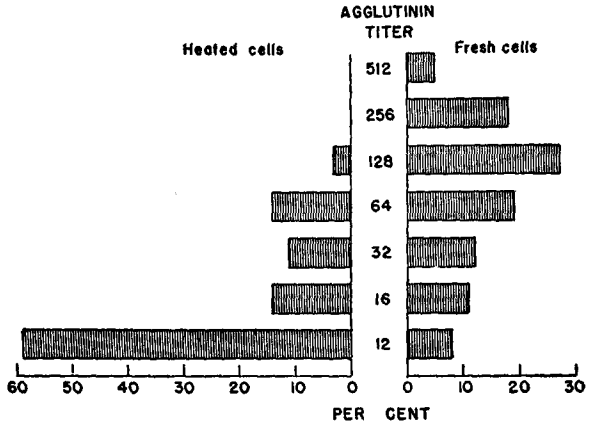

FIG. 3. Percentage frequency distribution of maximal agglutinin titers of non-irradiated recipients of fresh lymph node cells and of heated cells. The cells had been incubated *in vitro* with filtrates of trypsin-treated suspensions of dysentery bacilli.

in the subsequent serum titer of the recipient animals, and when the serum was added immediately after the filtrate was mixed with the cells there appeared to be a definite reduction in titer.

5. Use of Non-Irradiated Recipients. Comparison of Recipients of Fresh and Heated Cells.— In many experiments lymph node cells were transferred to normal as well as to irradiated recipients. It was found that after incubation of lymph node cells with filtrates of trypsin-treated suspensions of bacilli and transfer to normal recipients agglutinins appeared in the sera of most of the recipients on the 3rd or 4th day after transfer, reached a maximum level on the 6th to 8th day and then declined. The recipients of heated aliquots of these cell suspensions in many cases also developed agglutinins, which appeared on the 3rd, 4th, or 5th day after transfer. When antibody appeared in both these groups the titers of normal recipients of fresh cells were always higher than those of recipients of heated cells in the same experiment. Fig. 3 illustrates the percentage frequency distribution of the maximum titers of 93 recipients of fresh cells and 73 recipients of heated cells. More than 50 per cent of the recipients of heated

cells did not develop agglutinins above the threshold of measurement subsequent to transfer, whereas approximately 50 per cent of recipients of fresh cells developed titers of 128 or higher.

6. Comparison of Agglutinin Titers of Normal and Irradiated Recipients of Fresh Cells.— In experiments involving the transfer of aliquots of a given cell suspension to both irradiated and normal recipient rabbits it appeared that the irradiated recipients of fresh cells developed higher agglutinin titers than did normal recipients of fresh cells. The maximum titers of 148 irradiated and 82 normal recipients, obtained in the same experiments, were tabulated, and the geometric means were found to be 450 and 96, for irradiated normal rabbits, respectively. The percentage distribution of maximum titers for both groups of recipients is shown

TABLE V

Cytologic Observations on Suspensions of Lymph Node Cells Incubated in Vitro with Dysentery-Trypsin Filtrate and Transferred to Irradiated Rabbits

Donors	Cells sampled	No. of slides	Percentage distribution of cells													
			Lymphocytic series							Plasma cells		Monocytes		Eosinophils	
			Lymphocyte		Prolymphocyte		[1]Lymphoblast		Total						
			Range	Average	Range	Average	Range	Average	Average	Range	Average	Range	Average	Range	Average
Injected with heterologous antigen	Before incubation with antigen	5	90–93.6	91.4	4–8	6.1	0.5–2	1.3	98.8	0.7–1.5	0.74	0–0.7	0.14	0–1	0.3
	After incubation with antigen	7	90.5–97	92.3	2–9.5	5.4	0.3–2	1.5	99.2	0.5–1.5	0.7	0–0.5	0.2	0	0
Not injected	Before incubation with antigen	1	95	95	4	4	0.5	0.5	99.5	0.5	0.5	0	0	0	0
	After incubation with antigen	2	94–97	95.5	2–5	3.5	0	0	99.0	0–0.3	0.15	0.3–0.7	0.5	0.3	0.3

in Fig. 4. In these data the median value of peak titers of irradiated recipients was found to be more than two 2-fold steps higher than that of the non-irradiated recipients.

7. Cytologic Observations on Preparations of Transferred Cells.—In the experiments described above smears were made of the suspensions of lymph node cells at various stages of preparation. The smears were dried by a draft of cool air, fixed in methyl alcohol and stained with May-Gruenwald-Giemsa stain. Differential counts were made of 400 cells per slide. Since some donor rabbits in the cell transfer experiments had not been injected with any antigen and some had been injected with heterologous antigen, comparisons were made of the differential counts of cells of both sources. In addition, smears were made of the cell suspensions as they were collected and again after the

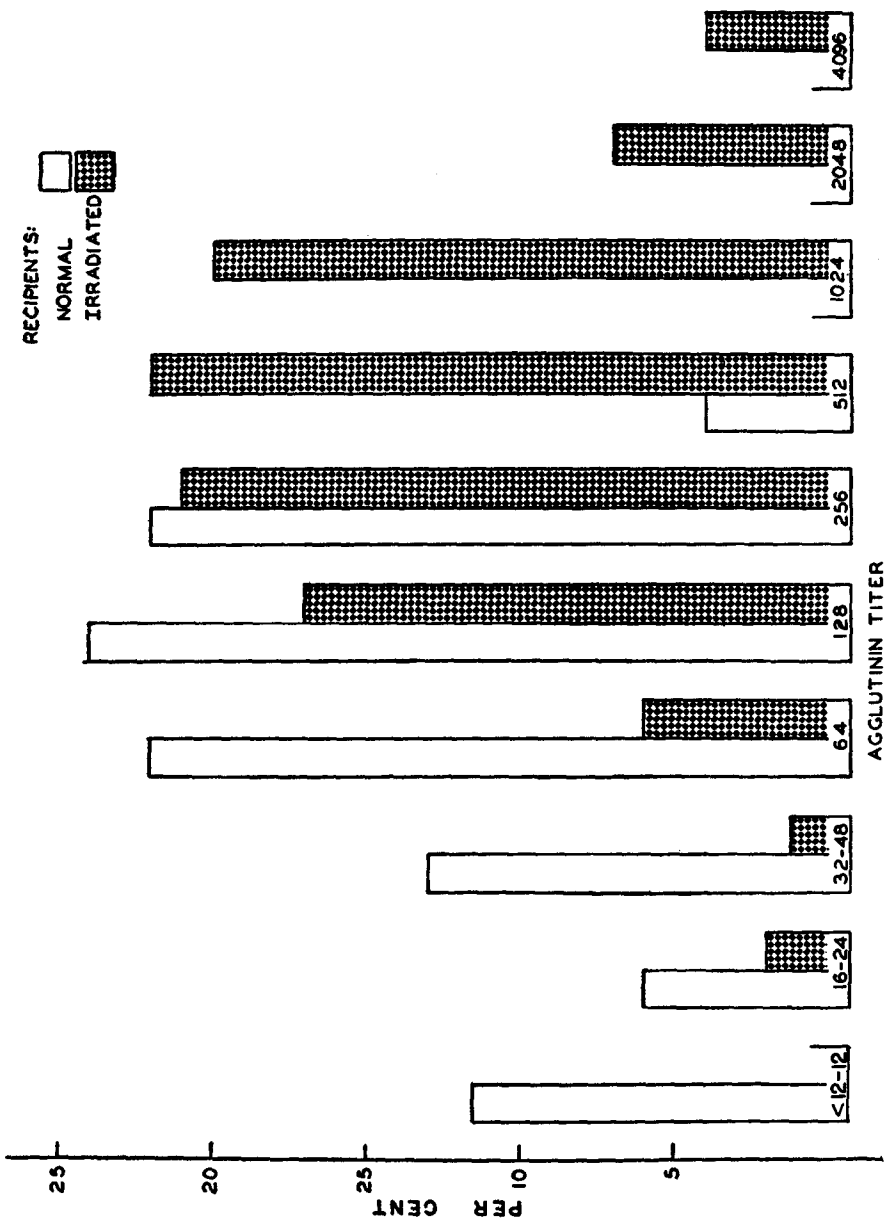

FIG. 4. Percentage frequency distribution of maximal agglutinin titers of irradiated and normal recipients of fresh lymph node cells incubated *in vitro* with *Shigella*-trypsin filtrate.

cells had been incubated *in vitro* with the filtrates of trypsin-treated dysentery bacilli. In Table V are shown both the range of percentage occurrence of the various types of cells and the average percentage for each type.

DISCUSSION

The Effect of Trypsin on Dysentery Bacilli.—The data reported above indicate that the filtrates of the trypsin-treated suspensions of *Shigella paradysenteriae* contained immunologically active material. This was demonstrated by various serologic tests using antiserum to whole *Shigella*, and also by the cell transfer experiments reported here, since the incubation with this material of lymph node cells from donor rabbits not previously injected with *Shigella* and the transfer of such cells to fresh irradiated rabbits was followed by the appearance in the sera of the latter of agglutinins to whole *Shigella*.

Filtrates of suspensions of *Shigella* in Tyrode's solution, at the same time and temperature as were used for incubation with the enzyme, showed some complement-fixing activity, as can be seen in Table III. However, that tryptic digestion of the bacilli *per se* yielded immunologically active material is indicated by three types of data:—

1. The ratios of complement-fixation titers of trypsin and control preparations shown in Table I were generally between 8 and 16, indicating that the percentage of serologically active material which was due to the effect of trypsin was of the order of 90 per cent of that found in the filtrates.
2. The serum agglutinin titers of recipients of transferred cells which had been incubated with filtrates of trypsin-treated suspensions were substantially higher than titers of recipients of cells incubated with control preparations, as was shown in Table III.
3. Increasing concentrations of complement-fixing material were found in filtrates of suspensions incubated with trypsin for longer periods or higher concentrations of the enzyme, as is indicated by the data shown in Table II.

In the cell transfer experiments such as those which provided the data presented in Tables I and III the lymph node cells were incubated with a dilution of 1:250 of the filtrates of suspensions of *Shigella*. In subsequent cell transfer experiments the maximal agglutinin titer of recipient rabbits was determined in relation to the concentrations of such filtrates used for *in vitro* incubation with lymph node cells. In experiments involving the use of the filtrates in a range of 1:250 to 10^{-4}, the mean peak titers of recipient animals were found to be similar. When lymph node cells were incubated with concentrations of the filtrate lower than 10^{-4}, however, the agglutinin titers of the recipients decreased correspondingly as is shown in Fig. 2, until, at 10^{-6} or 10^{-7} of the filtrate, little or no agglutinin was detectable in the sera of recipient rabbits.

In connection with this titration it is of interest to recall the data presented above on the dry weight per unit volume of such filtrates, *i.e.*, 1 mg./ml. or less. Since 1 ml. of such filtrates at a dilution of 10^{-4} gave a maximal level of antigenic stimulus to a suspension of 250 million lymph node cells, these data indicate that less than 10^{-4} mg. of the present antigenic material can produce substantial antigenic stimulus, with smaller degrees of effectiveness corresponding to one-tenth and one-hundredth this amount. The effective amounts of antigen are actually lower than those indicated, since no account has been taken of the amount of antigen not utilized by the lymph node cells during the *in vitro* incubation, or of the step of purification.

Such filtrates of trypsin-treated suspensions of *Shigella* yielded a preparation of antigen which proved to have several advantages. First, the concentration of the antigen in solution is fairly similar in different preparations as is indicated by the titers of recipient animals shown in Fig. 2. Second, the antigenic material is in substantially higher concentration than in the solutions obtained by the incubation of *Shigella* with normal rabbit serum as described in an earlier study (2). Another advantage of this preparation is that whereas the antigenic material is in solution for use in experimental manipulation, the homologous antibody can be measured by agglutination of the original bacteria, which makes possible relative measurements of antibody with the simplicity and high order of sensitivity of the agglutination test. Finally, and of most decisive advantage for studies of antibody formation, this preparation makes available a soluble antigen to which the primary antibody response is rapid and, relative to the sensitivity of the agglutination test, of adequate magnitude.

The Reaction in Vitro between Cells and Antigen.—Contact between cells and the soluble antigenic material was provided *in vitro*, and possibly to some extent in the tissues of the recipient host. It is considered likely, for two reasons, that some reaction between antigen and cells took place during the *in vitro* incubation. First, there is evidence that relatively little free antigen was transferred to the recipient animal in the cell suspension. A measure of such contaminating antigen is provided by the agglutinin titers of non-irradiated recipients given portions of heated cell suspension, since such animals are able to form antibody to the contaminating antigen with their own tissues, and the cell transfer effect is obviated by the heating of the cells. In the present study the sera of the majority of non-irradiated recipients of heated cells did not contain agglutinins above our threshold of measurement, as can be seen in Fig. 3, although almost all of the incubation involved in obtaining these data were with preparations of antigen at concentrations higher than 10^{-4}. As was described above, a concentration of 10^{-4} of filtrates was within the range which yielded maximal levels of agglutinin titer in irradiated recipients of fresh cells.

Other evidence for the occurrence *in vitro* of an initial reaction between cells and antigen is furnished by the data of experiments in which antiserum to *Shigella* was used as a reagent in the course of incubation of the cells. The concentration of this serum, relative to that of the filtrate used, was such as to provide a substantial excess of precipitating antibody to the soluble antigen(s) of the filtrate, as determined in preliminary experiments. This concentration of antibody, if incubated with the antigenic material before the incubation of the latter with lymph node cells, could prevent the reaction between antigen and cells to an extent such that recipients of cells so incubated developed very low agglutinin titers. However, the introduction of the antiserum at this concentration into the reaction mixture at any time after 5 minutes of incubation of cells and antigen alone did not affect the subsequent formation of agglutinins in the recipient rabbits. There is thus evidence that some reaction between lymph node cells and antigen occurs during the *in vitro* incubation, apparently within the first 5 minutes of such incubation.

Agglutinin Titers in Irradiated and Normal Recipients.—Among recipients of fresh lymph node cells which had been incubated with antigenic material, the irradiated animals showed higher maximal agglutination titers than the normal ones, the ratio being greater than four. Such differences have been observed also following the transfer to irradiated and normal animals of lymph node cells incubated with suspensions of whole *Shigella*, or with *Shigella*-treated serum. Similar differences were obtained in earlier work involving the transfer of cells from donor rabbits injected with *Shigella*. In a compilation, presented elsewhere, of data obtained in both donor injection experiments and *in vitro* incubation studies the difference between geometric means of maximal titers of irradiated and normal recipients was found to be quite similar in both systems (6).

Cytologic Observations.—The cytologic observations made on suspensions of transferred cells, which are summarized in Table V, indicate a great preponderance of cells of the lymphocytic series, the average percentages of these ranging between 98.8 and 99.5. A marked preponderance of cells of this series in suspensions of cells teased from lymph nodes has been observed by other investigators (7–9). It is also of interest that the percentages of lymphocytes, and of young forms within that series, were not substantially altered by the injection 4 days earlier of heterologous antigen. An understanding of the significance of these cytologic data in relation to the problem of the cellular source of antibody must await an elucidation of the role of the transferred cells in the formation of the antibody found in the recipient animals.

SUMMARY

Shigella paradysenteriae organisms were incubated in a solution of trypsin and then removed from the suspension by Seitz filtration. Serologic tests with

anti whole *Shigella* serum indicated that the filtrate contained antigenic material derived from the organisms. When lymph node cells from rabbits not previously injected with *Shigella* were incubated *in vitro* with such filtrates and then transferred to irradiated recipients, agglutinins to *Shigella* appeared in the sera of the latter. The transfer of heated suspensions of cells was not followed by the appearance of agglutinins in irradiated recipient rabbits. Non-irradiated recipients of lymph node cells incubated with the filtrate also developed agglutinin titers, to a lower level than irradiated animals. Agglutinins did not appear in the sera of the majority of non-irradiated recipients of heated cells.

The addition of excess antiserum to either the filtrate or the cell suspension before the incubation of these two materials resulted in a marked reduction in the subsequent agglutinin titers of recipient animals. However, if antiserum was added at the conclusion of the usual 30 minute incubation of cells and filtrate, or even after 5 minutes of incubation, there was no reduction in the agglutinin titers of the recipients.

Cytologic examination of aliquots of a number of the suspensions of cells prepared for transfer revealed that approximately 98 to 99 per cent of the cells belonged to the lymphocytic series.

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