known, although toxic or ischemic effects are considered to be the most probable explanation.

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Nature of Leukocyte Agglutinating Agent Occurring in Blood of Immunized Animals.* (24841)

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Agglutination of blood leukocytes occurs in a variety of apparently unrelated phenomena (1), among which are *in vivo*(2-4) and *in vitro*(5) hypersensitivity reactions. Such agglutination is probably a manifestation of cell injury, or at least an alteration at cell surface. In hypersensitivity reactions, agglutination would be due to a reaction of antigen with an agent probably antibody. Characterization of such an agent is a prerequisite to studying the mechanism by which an antigen-antibody reaction alters leukocytes so that they agglutinate. The work here presented was undertaken to determine whether the leukocyte agglutinating agent occurring in blood of immunized animals behaves in a manner characteristic of antibody and whether such antibody is cellular or humoral.

Material and methods. Male guinea pigs weighing 400-500 g were used. Animals in experimental group received a single intraperitoneal injection of either 1 mg crystalline egg albumin (Armour) in 1 ml normal saline or 1 ml heat killed brain-heart infusion broth culture of type III pneumococci (ATCC 6303) containing approximately 10^5 organisms/ml. Control animals received a single intraperitoneal injection of either 1 ml saline or 1 ml sterile brain-heart infusion broth. Animals which received egg albumin (EA) and their controls were used 6-10 weeks following injection. Animals which received pneumococcal vaccine and their controls were used after 8-12 weeks. Blood was obtained by cardiac puncture. Serum was stored at –30°C after separating from clotted blood which remained at room temperature not more than 2 hrs. Solutions of EA, pneumococcal capsular polysaccharide (S III)† and heparin (.3 mg/ml) were prepared with normal saline. Balanced salt solution contained isotonic concentrations of NaCl and KCl. All solutions in contact with leukocytes were pyrogen free. Leukocyte agglutination was quantitated by technic previously described(6) and degree of agglutination expressed as agglutination index. The *t* test was used for all statistical evaluations. Two methods were used to test for leukocyte

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agglutinating antibody (LAA). (a) The *first* method consisted of serum-cell cross experiments involving cells from one animal and serum from a second in the following systems: nonimmune cells + nonimmune serum, nonimmune cells + immune serum, immune cells + nonimmune serum and immune cells + immune serum. Three ml of blood were drawn into heparin moistened syringe and immediately discharged into 30 ml balanced salt solution rendering blood uncoagulable. Formed elements were separated from supernate by centrifugation at 1000 rpm and washed x 3 with additional 10 ml portions of balanced salt solution by resuspension and centrifugation. The washed formed elements were resuspended in 1.5 ml of immune or nonimmune serum according to experimental design. To .45-ml samples of serum-cell mixture .05 ml of either saline or a dilution of antigen was added, the samples incubated for 4.5 min. at 37.5°C, gently shaken for 30 sec. and agglutination indices determined. In each experiment a blood sample containing saline served as internal control for nonspecific leukocyte agglutination. (b) The *second* method involved transfer of leukocyte agglutinating activity to nonimmune blood by adding immune serum. To .40-ml samples of normal blood .05-ml volumes of diluted immune serum were added and, after adding .05-ml volumes of specific antigen at appropriate concentration, agglutination indices were determined. In each experiment a blood sample containing saline instead of immune serum served as internal control. To determine titer of LAA in immune sera the second method was used by adding different dilutions of immune sera to normal blood. In testing heat stability of LAA, nonheated immune serum and a sample of the same serum heated at 56°C for 30 min. were added to normal blood and LAA activity determined by second method. Hemagglutination and anti-EA adsorption were carried out with tannic acid treated human O, Rh positive red cells according to Stavitsky(7). Tanned cells were treated with either EA or saline as control and in certain experiments with bovine serum albumin or bovine gamma globulin (Armour). Sera to be adsorbed were from EA injected animals and demonstrated leukocyte agglutinating activity. Hemagglutination titers were read after 2 hrs. at room temperature and again after overnight refrigeration. Supernatant sera were then removed from certain tubes in which hemagglutination had occurred and their leukocyte agglutinating activities tested by second method of testing for antibody. Pneumococcal adsorption of sera from animals injected with type III pneumococci and showing leukocyte agglutinating activity in the presence of S III was done with both type I and III pneumococci. Pneumococci were cultured in 500 ml Todd-Hewitt broth(8) for 18 hrs. and then killed by heating at 56°C for 1 hr. Killed bacteria were centrifuged, washed twice with 20 ml normal saline and final packed bacteria divided into 5 equal portions. Each portion was used to adsorb a .5-ml sample of serum for 1 hr. at room temperature. The adsorbed sera were collected from pneumococci after centrifugation and their leukocyte agglutinating activities tested by second method.

Results. Table I shows results of cell-serum cross experiments. Each value is average of 10 experiments. The minimum antigen concentration increasing leukocyte agglutination over the saline control in the immune serum + immune cell system was .1 μg/ml for EA and .01 – .1 μg/ml for S III. In the nonimmune serum + nonimmune cell system it was 10 μg/ml for EA and .5 – 1.0 μg/ml for S III. That these antigens cause leukocyte agglutination in normal blood at higher concentrations than in immune blood has been reported(5). When nonimmune cells were suspended in immune serum, antigen concentrations characteristic of immune cell + immune serum system resulted in significant increase in agglutination, whereas, when immune cells were suspended in nonimmune serum, antigen concentrations characteristic of nonimmune cells + nonimmune serum system were required. Significant increases in agglutination indices over saline controls were small compared to previous observations(5) and were due to smaller workable cell population which resulted from cell disruption during experimental manipulation. However, these sig-

TABLE I. Demonstration of Leukocyte Agglutinating Factor in Serum from Immune Animals.

Avg difference of agglutination index from saline control ± S.E.																		
Immune serum				Nonimmune serum				Immune serum				Nonimmune serum						
" cells				" cells				Nonimmune cells				Immune cells						
Egg albumin, μg/ml			SIII, μg/ml	Egg albumin, μg/ml			SIII, μg/ml	Egg albumin, μg/ml			SIII, μg/ml	Egg albumin, μg/ml			SIII, μg/ml			
.01	.1	1.0	.01-.1	.1	1.0	10	.01-.1	.5-1.0	.01	.1	1.0	.01-.1	.1	1.0	10	.01-.1	.5-1.0	
1.8 ± 1	5.5 ± 1.5	7.8 ± 1	5.0 ± .6	.1 ± .8	1.2 ± .8	4.0 ± .9	.2 ± .8	5.9 ± 1	.9 ± .8	4.1 ± .8	7.2 ± 1.1	4.6 ± .6	.0	.0	5.5 ± 1.1	1.5 ± .9	4.2 ± .6	
P = .1	P < .01	P < .01	P < .01	P = .9	P = .15	P < .01	P = .8	P < .01	P = .2	P < .01	P < .01	P < .01			P < .01	P = .15	P < .01	

nificant differences represent 58-90% increases over the saline control.

Table II shows that immune serum, when added to normal blood, caused an increased leukocyte agglutination in the presence of specific antigen. Agglutination indices for blood samples containing saline are compared with samples containing immune serum. The highest effective serum dilution is given. Table II also shows that heating such sera before they were added to normal blood destroyed their leukocyte agglutinating activity.

Tables III and IV show adsorption of humoral antibody by specific antigen. Table III indicates EA tanned red cells agglutinated in the presence of anti-EA sera, whereas, saline, bovine serum albumin and bovine serum gamma globulin treated tanned cells did not. When such sera were removed from agglutinated red cells, added to normal blood and their leukocyte agglutinating activities determined in presence of specific antigen, the leukocyte agglutinating activities of sera from only EA tanned cells were diminished (Table III). Table IV shows composite results of 10 experiments in which sera from animals receiving type III pneumococci were adsorbed with both type I and III organisms and their leukocyte agglutinating activities tested. It is apparent that only type III organisms diminished the leukocyte agglutinating activity of such sera.

Discussion. Previous work has shown that, although LAA can be demonstrated in blood of animals susceptible to anaphylactic shock, the anaphylactic type of hypersensitivity is not a prerequisite for its presence(5). Likewise, LAA can be demonstrated in serum of animals without detectable precipitating antibody(5). This suggests that either the LAA and precipitating antibody are different or the technic for detecting classical antibody by leukocyte agglutination is relatively more sensitive than usual methods for detecting precipitating antibody. The work here presented has not attempted to answer this question, but to determine whether the LAA is humoral or cellular and whether it behaves as an antibody. That LAA is humoral has been shown by 3 types of experiment. Cell-serum cross ex-

TABLE II. Titers of Immune Sera Producing Leukocyte Agglutination in Normal Blood with Specific Antigen. Effect of heat on leukocyte agglutinating activity of such sera.

	Agglutination index				Agglutination index		
Exp.	Saline	Serum	Serum titer	Exp.	Saline	Untreated serum	56°C serum
EA 1	57	75	1/500	EA 7	16	24	11
2	28	48	"	8	17	23	20
3	53	66	1/1000	9	31	52	28
4	21	31	1/500	10	24	33	23
5	14	40	1/1000	11	21	38	20
6	41	54	"				
SIII 1	8	15	"	SIII 9	15	24	17
2	9	17	1/500	10	31	46	35
3	12	18	1/1000	11	43	69	40
4	1	6	"	12	17	28	19
5	1	9	"	13	28	45	23
6	1	7	1/5000	14	27	42	27
7	4	13	1/500				
8	4	11	"				

Final antigen concentration: EA, 1 μg/ml; SIII, .1 μg/ml.

periments have shown that nonimmune cells + immune serum react at antigen concentrations which characterize an immune system, whereas, immune cells + nonimmune serum react at antigen concentrations characterizing a nonimmune system. LAA can be transferred *in vitro* to normal blood by adding small amounts of immune serum and such activity can be destroyed or impaired by heating such sera at 56°C. Finally, LAA can be specifically adsorbed from immune serum. It must be concluded that with the experimental model used there was no evidence for cellular antibody.

There is also convincing evidence that the humoral agent producing leukocyte agglutination behaves as an antibody. Immunization resulted either in formation of a new unit capable of reacting with antigen or increased the number of preexisting units capable of reacting with antigen above a critical threshold, which was adequate to trigger leukocyte agglutination in the immune system. Also, from Tables III and IV, the humoral factor is shown to have an affinity for specific antigen. The factor occurring in anti-EA sera was adsorbed by EA tanned red cells but not by tanned cells treated with saline, bovine serum albumin or bovine gamma globulin. Similarly, the factor occurring in anti-type III

TABLE III. Effect of Adsorption by Specific Antigen on Leukocyte Agglutinating Activity of Immune Serum.

	Reciprocal hemagglutination test titers				Leukocyte agglutination index (EA, 1 μg/ml)					
Serum	EATC	STC	BGGTC	BSATC	Saline	Non-adsorbed serum	STC serum	EATC serum	BGGTC serum	BSATC serum
EA 1	4096	Neg.			31	65	60	44		
4	1024	"			9	29	26	8		
7	4096	"			26	45	39	24		
8	4096	"			15	lost	27	11		
9	8192	"			8	19	16	6		
11	16,384	"	Neg.	Neg.	20	28	25	21	27	26
12	2048	"			27	31	34	20		
13	16,384	"	"	"	6	11	12	6	12	13
16	128	"			38	106	102	74		
17	4096	"			50	60	65	47		
Normal	Neg.	"								
SIII	"	"								

(EATC) egg albumin tanned cells; (STC) saline tanned cells; (BGGTC) bovine gamma globulin tanned cells; (BSATC) bovine serum albumin tanned cells.

TABLE IV. Difference of Average Agglutination Index Due to Adsorption of SIII Immune Serum by Type I and Type III Pneumococci.

Significance between	Difference ± S.E.	P
Saline and nonadsorbed serum	6.2 ± .8	<.01
Saline and Type III adsorbed serum	1.1 ± 1.0	.30
Nonadsorbed serum and Type III adsorbed serum	7.3 ± 1.2	<.01
Saline and Type I adsorbed serum	8.4 ± 1.2	"
Type I adsorbed serum and Type III adsorbed serum	9.5 ± 1.4	"

SIII, .1 μg/ml.

pneumococcal sera was adsorbed by type III pneumococci but not by type I. From the work of Borduas and Grabar(9) it may be questioned whether the EA tanned cells really adsorbed anti-EA antibody, since these workers claim that tanned cells preferentially adsorb conalbumin rather than EA. Stravitsky has shown that EA is adsorbed about as well as conalbumin(10). The fact that this issue may be controversial does not invalidate the results of our study. Whether EA or one of its impurities was the principal antigenic unit. LAA was formed and adsorbed from serum by antigenic unit fixed to tanned red cells.

Waksman reported that rabbit anti-ovalbumin sera produced a decrease in leukocyte count when added to normal blood in presence of specific antigen(11). He found no correlation between white cell lytic activity of such antisera and precipitating antibody but did find some correlation with skin sensitizing antibody. It is likely that the antibody involved in decreased leukocyte count observed by Waksman and the LAA are identical. However, the nature of LAA and its exact relation to antibody of known type remain to be elucidated.

Summary. A humoral agent produced by single injection of antigen and responsible for leukocyte agglutination, which occurs in presence of specific antigen has been demonstrated in anti-EA and anti-type III pneumococcal sera. The factor is heat labile and, due to its specific affinity for EA treated tanned red cells and type III pneumococci respectively, it is thought to be antibody.

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Histochemical Demonstration of Erythrocyte Esterases.* (24842)

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Although the histochemical demonstration of esterases in tissues can be performed by means of azo and indoxyl technics, such technics have not been applied successfully to erythrocyte esterases(1). The demonstration of acetylcholinesterase, based on the Koelle method, was noted by Zajicek *et al.*(2); the precipitate, however, is crystalline and localization of enzyme activity within the erythrocyte has not yet been reported. During preliminary studies on development of high resolution histochemical technics for intracellular localization of enzymes, erythrocyte esterase activity could be demonstrated with excellent localization in ordinary air-dried blood smears.

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