IMMUNOLOGIC STUDIES WITH ETHYLENE OXIDE-TREATED HUMAN SERUM*, ‡

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Plates 105 to 107

(Received for publication, February 2, 1961)

One of the main problems associated with the sterilization of plasma and plasma fractions is the inactivation of the serum hepatitis virus. Ideally, this should be accomplished with the minimal alteration of the serum proteins. If the extent of alteration of these proteins is too great, the possibility that they may become antigenic in man must be considered. Ethylene oxide gas which has been used as a sterilizing agent for foods, antibiotics, and medical plastics has been considered for the sterilization of bacteria and viruses. The basic reaction is an alkylation of reactive groupings in the protein such as lysine and histidine. The data presented in the present report deal with antigenicity studies in humans and rabbits of several ethylene oxide–treated human sera. As will be shown, definite "delayed cellular hypersensitivity" reactions were produced in humans.

Materials and Methods

The ethylene oxide (E-O)–treated materials were supplied by Courtland Laboratories, Los Angeles, California and will be referred to as CLM-treated sera. A special preparation, 500S, for injection into humans, was obtained from a donor known to be free of serum hepatitis.

After withdrawal of 50 ml. of blood, the volunteers were skin-tested with 0.1 ml of the different materials, and then injected intramuscularly 5 times over a 10 day period with solutions of various amounts of the CLM-treated serum. The bleeding and skin testing were repeated 10 days and 3 weeks after the last injection. The serum was stored in the cold with 1:10,000 "merthiolate" and 0.25 per cent phenol as preservatives. The skin reactions were read after 15 to 20 minutes and after 24 hours. The pre- and postimmunization sera were analyzed by several *in vitro* and *in vivo* procedures.

The agar diffusion techniques of Preer (1) and Ouchterlony (2) employing undiluted antiserum and varying dilutions of the antigen (undiluted, 1–10, 1–100, 1–1000, 1–10,000, and 1–100,000) were set up at 25° and at 0°C.

* Supported by the Department of the Army, Office of the Surgeon General, Contract Nos. DA-49-007-MD-248 and DA-49-193-MD-2113 and United States Public Health Service Grants E 1268 and E 3514.

‡ Presented in part at the Annual Meeting of the American Association of Immunologists, Chicago, Illinois, April, 1960.

The microprecipitin technique for analysis of human sera as developed by Heidelberger and MacPherson (3) using 3.0 ml. of serum and 1.0 ml. of the indicated dilutions of CLM-treated serum was set up at 0° and observed for 14 days before analyzing the mixtures.

For studies of passive systemic anaphylaxis guinea pigs weighing 200 to 250 gm. were given intraperitoneal injections of 1, 2, 4, or 5 ml. of the serum obtained from volunteers 105 and 111. 48 hours later, the guinea pigs were injected intravenously with 2 ml. of the CLM serum, and observed for signs of anaphylaxis.

The sensitive passive cutaneous anaphylaxis test (PCA) of Ovary (4) was performed in guinea pigs weighing about 250 gm. Guinea pigs were given an intracutaneous injection of 0.1 ml. serum obtained from the various bleedings. 3 hours later, the guinea pigs were given an intravenous injection of 1.25 ml. of CLM serum and dye (1 ml. serum and 0.25 ml. 1 per cent Evans blue dye). Any blueing of the injected sites was recorded.

Biopsies of immediate and delayed skin reactions of donors and recipients[1] were obtained by sterile surgical procedures and fixed in formalin for 24 hours before histological sectioning and staining with hematoxylin and eosin.

The transfer of the delayed hypersensitivity reactions by viable and killed leukocytes was performed according to the procedures outlined by Lawrence *et al.* (5). Leukocytes harvested from 100 ml. of peripheral blood of the 2 best reactors were injected subcutaneously in a volume of 1 to 2 ml. into the forearm of normal recipients. 24 hours later the recipients were skin-tested on the opposite forearm. Skin reactions were observed for 72 hours and some biopsies obtained after 24 hours. Extracts of leukocytes were obtained by freezing the cells in a dry ice–acetone mixture and thawing them at 37°C. The above was repeated 7 times, and the cell extracts centrifuged in the cold before injection into volunteers.

Six male albino rabbits weighing about 2.5 to 3 kg. were immunized with a complete adjuvant mixture of the CLM 500S preparation containing 25 mg. protein per ml. The mixture contained equal parts of serum and equal volumes of a mixture of bayol F (9 parts) and arlacel C (1 part) (6, 7). Dead mycobacteria (1 mg. per ml.) were also included. The first injection of 1 ml. was made in the hind foot-pads. 2 weeks later 1 ml. of adjuvant was injected intramuscularly in the hind quarter. The rabbits were bled weekly, beginning 3 weeks after the last injection. The sera were tested qualitatively and the high titered sera were pooled. The rabbit antisera were studied by several agar diffusion techniques based on the Ouchterlony method. Dilutions of the CLM serum (1–10, 1–100, 1–1000) and normal human serum were reacted with the anti-CLM 500S serum in veronal-glycine-agar medium (8). In additional studies, the agar was made up to contain 25 per cent normal human serum (9). The center wells were filled with antisera and the outside wells with dilutions of serum or plasma.

Rabbit antisera against human serum albumin and human gamma globulin were the same as used in previous publications (10). They were prepared by intravenous injections of alum-precipitated proteins. The antisera were calibrated by the quantitative immunochemical techniques developed by Heidelberger and coworkers (11). Nitrogen content of all samples was determined by the Markham modification of the micro Kjeldahl method (12).

RESULTS

Table I presents the data on the skin reactions observed before and 10 days after immunization of individuals injected with different amounts of CLM-treated serum. Some individuals showed positive "skin reactions" to the materials before immunization. The reactions were slightly erythematous and

[1] The author wishes to thank Dr. William White of the University of Pittsburgh, School of Medicine, and his staff for performing the surgical biopsies.

had some speckled areas. The reactions were gone in 1 to 2 hours, and did not return the following day. After the fourth injection, several individuals had flare-up reactions at the initial skin test site of the CLM serum (Fig. 1). The dotted markings indicate the size of the erythema the night before the reaction was recorded. Other observations noted during the immunization were as follows: Volunteer 104 observed that the day after the last injection, the original CLM serum site was itching and measured 9 mm. in diameter. Volunteer 105 reported that 4 days after the last injection, the skin test site was warm and measured 21 × 40 mm. Volunteer 108 reported an erythema 16 mm. in diameter the day after the last injection. Volunteer 111 reported that 4 hours after the last injection, the original skin test site had flared up to 20 mm. diameter. It was red and itching. The results of the skin tests observed 10 days after the last injection indicated that the CLM serum was inflammatory and produced some immediate reactions which faded rapidly. After 2 to 4 hours there was a gradual return of a positive reaction to the CLM material. This sort of behavior was repeated at each time of testing for 1½ years and is shown again with the data obtained at the 3 week testing period. (Table II). The type of delayed reaction usually observed is shown in Fig. 2.

TABLE I

*Results of Skin Testing with CLM Serum**

Volunteer No.	CLM serum injected per injection	Preimmunization		10 days postimmunization					
				CLM serum			0.15 M NaCl		
		CLM serum	0.15 M NaCl	Immediate	24 hrs.	48 hrs.	Immediate	24 hrs.	48 hrs.
	mg.								
104	5	0	0	22	23	25	0	3	0
105	5	10‡	0	20	32	50	5	5	0
106	5	0	0	24	0	10	0	0	0
107	5	30‡	0	40	8	0	0	0	0
108	10	20‡	0	5	22	30	0	5	0
109	10	30‡	0	22	24	x	10	10	0
110	10	22‡	0	18	20	0	10	0	0
111	10	15‡	0	20	30	35	10	0	0
112	25	30‡	10‡	25	18	0	10	0	0
113	25	0	0	31	15	0	10	0	0
114	25	25‡	8‡	17	xx	20	10	xx	0
115	25	13‡	0	30	15	0	10	0	0

x, large red swollen area.

xx, not read.

* All values given in millimeters (diameter of reaction); all reactions were erythematous.

‡ Very pale reaction.

The most sensitive reactors were tested with 1–100 and 1–10 dilutions of the CLM serum as well as with undiluted material. The behavior was always the same, *i.e.*, an immediate reaction which was gone in 1 hour, followed by a gradual return of the delayed type of reaction which was visible after about 6 hours. The immediate reaction appears to be related to the fact that the CLM serum behaved as a "histamine liberator." This was observed by injecting guinea pigs intravenously with Evans blue dye and then injecting 0.1 ml. of the CLM serum intradermally. Definite blueing reactions occurred with serum dilutions as low as 1: 10,000.

TABLE II

*Results of Skin Testing Volunteers with CLM Serum 3 Weeks after Last Injection**

Volunteer No.	Dilution injected intradermally	CLM serum		0.15 M NaCl	
		Immediate reaction	24 hr. reaction	Immediate reaction	24 hr. reaction
104	1–10	10	20	0	2
105	1–100	0	20	0	0
106	Undiluted	25	25	0	0
107	"	40	5	0	0
108	1–10	0	15	0	0
109	1–10	25	25	0	0
110	Undiluted	30	15	10	0
111	1–100	25	13	0	0
112	Undiluted	35	25	5	0
113	"	30	0	5	0
114	"	35	10	0	0
115	"	40	0	0	0

* All values given in millimeters (diameter of reaction) of erythema reaction.

Biopsies of the early and 24 hour reaction skin test sites were examined histologically.[2] The immediate reaction was mostly of an acute inflammatory nature and consisted mainly of polymorphonuclear cells. The 24 hour reaction shown in Fig. 3 indicates a heavy perivascular lymphocytic infiltration without vessel wall damage. This same sort of behavior was present in biopsy sites of other volunteers. The immediate type reaction interfered with the interpretation of the results obtained in Prausnitz-Kustner reactions. $\frac{1}{10}$ ml. of various bleedings from the sensitive individuals was injected intradermally into the forearm of normal individuals. 24 hours later these sites, as well as uninjected areas of the opposite forearm, were challenged with dilutions of the CLM

[2] The author wishes to thank Dr. J. J. Vazquez of the University of Pittsburgh Medical School for the interpretation of the histological sections.

serum.[3] No differences in skin reactions were observed between the sensitized and the unsensitized areas. When the sera from the sensitized individuals were analyzed for antibody by the *in vitro* and *in vivo* methods mentioned above, none was detectable.

Attempts to transfer this delayed cellular sensitivity to normal recipients were successful with either viable leukocytes or extracts of the lymphocytes. When the recipients of the cells or extracts of cells were challenged intradermally they exhibited a reaction pattern similar to that of the sensitive donors,

TABLE III

Skin Reactions in Normal Recipients of Leukocytes from Sensitive Donors, ‡*

Volunteer No.	State of leukocytes	Recipient	Time of reaction			
			Immediate	4 hrs.	24 hrs.	48 hrs.
105	Dead	G.O.	15	12	35	40
105	"	R.F.	30	20	35	20
111	"	A.N.	35	15	20	0
111	"	M.R.	25	11	30	0
111	Dead	T.T.	35	10	20	0
			0§	0§	16§	10§
105	Viable	C.C.	15	10	30	15
105	"	J.M.	25	15	40	20
111	"	L.M.	20	10	35	20

* Recipients received leukocytes harvested from 100 ml. of whole blood.

‡ All reactions reported are diameter of erythema after injection of 0.1 ml. of undiluted CLM serum.

§ Observations in tuberculin-negative recipient after transfer of leukocyte extracts. Purified protein derivative used for skin testing.

i.e., a transient immediate reaction followed by a return of a delayed reaction. Table III gives the detailed data obtained with several recipients. In addition to the transfer of sensitivity to the CLM serum, sensitivity to tuberculin (PPD) was transferred to one recipient. The donor was tuberculin-positive and the recipient was tuberculin-negative before the transfer of the cells.

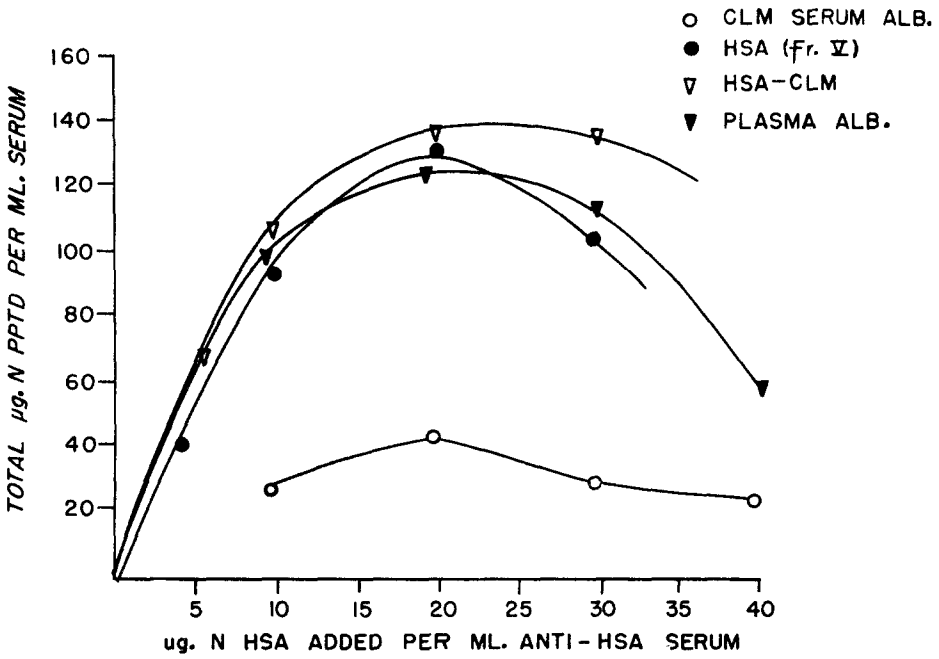
The histological appearance of the 24 hour reaction area is shown in Fig. 4. Here too, there is mainly a perivascular lymphocytic infiltration.

The results mentioned so far would indicate that we are dealing with a definite immune response in humans against ethylene oxide–treated human

[3] The recipients of the serum injections were skin tested a year later and it was noted that they had become "delayedly hypersensitive" from the intradermal injections.

serum. The type of reaction is certainly compatible with the tuberculin, cellular, or delayed reaction. Several manifestations of the reaction such as (*a*) absence of detectable antibody, (*b*) time of appearance of gross reaction, and (*c*) the histological appearance of the sites, all point towards a true delayed type reaction.

The results of the immunochemical studies with antisera against the CLM-treated human serum indicate that extensive alteration occurred in the serum



TEXT-FIG. 1. Homologous and cross-precipitation reactions with rabbit anti-human serum albumin serum (dilution 1–5). ○, CLM serum albumin in CLM 500S serum; ●, Normal human serum albumin (fraction V); ▽, Normal human serum albumin treated mildly with ethylene oxide; ▼, Serum albumin in normal human plasma preparation.

proteins during the sterilization procedures. Fig. 5 shows the expected multiplicity of antibodies produced against the 500S CLM serum. When the diffusion in agar was performed in the presence of normal human serum two distinct bands still appeared (Fig. 6). This would indicate that at least 2 entirely new modified serum proteins were present in the preparation. The extent of their alteration was such that the rabbit antibodies against them did not react with normal human serum. That extensive alteration did occur was also shown by the decreased reactions of the CLM serum albumin with rabbit antisera prepared against human serum albumin (Text-fig. 1), and human gamma globulin. The HSA-CLM in the figure refers to human serum albumin that was

treated with E-O under mild conditions; the plasma albumin curve was obtained with appropriate dilutions of a normal untreated human plasma preparation.

DISCUSSION

The present investigation was undertaken mainly to learn whether a certain ethylene oxide–treated human serum could be antigenic in man. Although it has been shown that chemically modified proteins can be antigenic in the homologous animal (13), the extent of modification was assumed to be minimal in this preparation. Earlier preparations of E-O–treated serum and plasma did not show any change in electrophoretic behavior and did not produce any reactions upon infusions into man (14). However, the extent of modification in this special serum 500S due to alkylation of lysine and histidine groups was extensive. This modification was confirmed by the poor reactions of the 500S serum with antiserum produced against human serum albumin and human gamma globulin.

When several other sera and plasmas were studied by reaction with the anti-CLM 500S serum in serum-agar plates, 3 out of 7 gave a positive reaction. This would indicate that if any method is proposed for sterilization of plasma, it should be completely standardized before testing in humans. Another important point of this study is that in addition to analysis of the serum for antibody, skin testing of the subjects should always be performed. In the absence of skin testing, there would have been little reason to doubt the utility and non-antigenicity of the preparation.

It was also realized that it may be worthwhile to study the effect of the ethylene oxide treatment on other sera such as guinea pig serum. When guinea pigs were injected with extensively modified guinea pig serum, observations similar to those noted with the humans were made. However, in addition to the delayed type of reactions, there were also several true immediate reactions. This latter observation at present appears to be related to the allotypic forms of guinea pig gamma globulin and will be reported separately (15). Similar data on the antigenicity of guinea pig gamma globulin in guinea pigs have recently been obtained by Benacerraf and Gell (16). The possibility that the delayed type skin reactions reported here could be ascribed to an immune response against the allotypic forms of human gamma globulin as has been considered previously (17) was ruled out here. In the study reported here 9 out of 12 individuals gave positive delayed skin reactions after immunization whereas in the previous study 1 individual out of 57 injected with heat-treated human plasmas showed a positive delayed skin reaction with the concentrated protein solutions. This reaction was not present when the individual was tested with the 1–10 and 1–100 dilutions of the antigen.

The delayed cellular reactions in the absence of detectable circulating

antibody observed in the humans and guinea pigs confirms the reports of Benacerraf and Gell (18, 19). They immunized guinea pigs with picrylated guinea pig globulin or acetylated guinea pig globulin and noted marked delayed reaction in the absence of circulating antibody. In the papers of these latter workers, (18–20) as well as those of Pappenheimer (21), and Salvin (22, 23), the question of whether cellular immunity is a stage in antibody production has been discussed. From the results presented here with humans, this did not seem to be the case. Repeated injections over a period of 1½ years of the modified serum proteins did not produce detectable circulating antibody in the most exquisitely sensitive individuals. From this single observation, one cannot generalize, but it would appear that in humans it is possible to have delayed sensitivity persist in the absence of circulating antibody. This is certainly easier to produce with homologous serum proteins rather than with heterologous proteins as discussed by Benacerraf and Gell (19).

It is difficult to explain the cause of the transient immediate reactions in both humans and guinea pigs. The material which caused "histamine" liberation and change in vascular permeability is unknown. However, from the work of Ishizaka and Ishizaka (24) it appears that aggregated human gamma globulin prepared by various procedures can induce skin reactions in normal guinea pigs similar to those produced by soluble antigen-antibody complexes. As suggested by these workers, the change in molecular configurations of the gamma globulin may be responsible in part for the biologic activity. Physiochemical and immunochemical studies of the CLM 500S preparation have indicated the presence of aggregated and altered gamma globulin which may account for the odd immediate inflammatory reactions.

It has been suggested by Waksman (25, 26) that many autoimmune reactions fall into the classification of delayed cellular reactions. One of the explanations advanced to account for the immune responses is that there is some change in the structure of the tissue protein, leading to the production of a protein foreign to the host. The experiments of Benacceraf and Gell and the ones reported here would support the concept that delayed reactions in the absence of antibody can be produced against modified homologous proteins.

From the histological pictures presented in Figs. 3 and 4, the delayed reactions appear to consist of an almost pure mononuclear cell infiltration. This, as well as the initial polymorphonuclear cell predominance would make the observed reaction compatible with the tuberculin type of reaction. Although intense reactions were observed in many individuals, at no time was there any necrosis or tissue damage.

The transfer of the sensitivity to normal donors by extracts of sensitive leukocytes confirms Lawrence's observations on the transfer in man with other systems such as diphtheria toxin (27), tuberculin (5), and streptococcal proteins (28). However, in these latter reports, the question was always raised

whether the recipients may have been sensitized by the pre–cell transfer skin testing. In the experiments reported here, the recipients were never skin-tested until after the transfer. Moreover, the recipients could not have been exposed by any natural means to the E-O–treated material before skin testing. These experiments seem important in supporting the investigations of Lawrence and extending them to include other materials in addition to those of bacterial and fungal origin (29). It is also of some interest that the recipients were still delayedly hypersensitive a year after the initial transfer and skin testing.

SUMMARY

The antigenicity of an ethylene oxide–treated human serum in humans has been studied. The immune response to the material had many of the characteristics of a delayed cellular skin reaction. Even after repeated immunizations by intradermal skin testing over a period of 1½ years, no detectable antibody could be found in the sera. The antigenicity has been shown to be associated with drastic alteration of the homologous serum proteins as evidenced by (*a*) the formation of new proteins, and (*b*) the poor cross-reactions of the modified serum proteins with antisera against normal human serum albumin and normal human gamma globulin.

The delayed hypersensitivity was transferable to normal recipients by either viable or killed leukocytes. The implications of these findings have been discussed with respect to the problem of sterilization of sera, the tuberculin reaction, and autoimmune phenomena.

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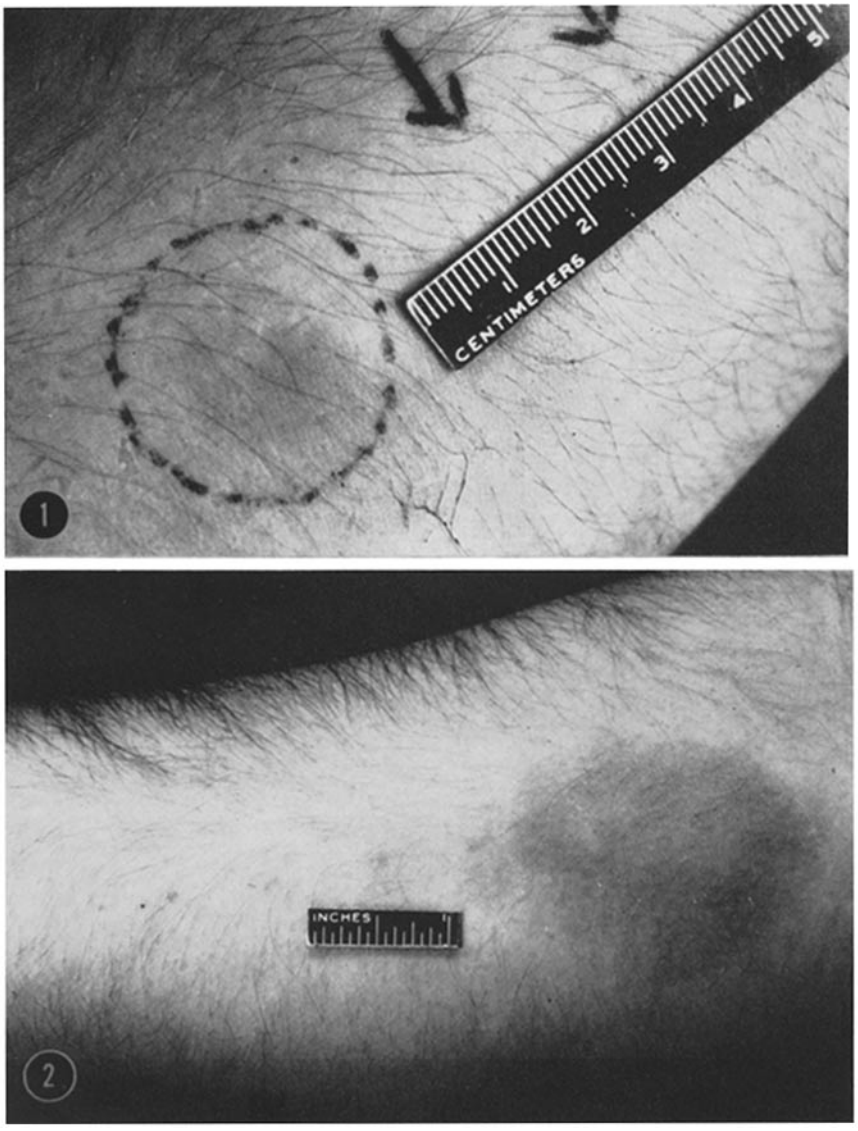
EXPLANATION OF PLATES

PLATE 105

FIG. 1. Flare-up reaction at initial skin test site observed in volunteer 105 1 week after skin testing. Dotted area refers to size of reactions the evening before recording. Arrows indicate other skin test sites of 0.15 M NaCl and 5 per cent human serum albumin.

FIG. 2. Skin reaction observed 24 hours after testing with CLM serum. Note four other negative reactions (normal human serum, 5 per cent human serum albumin, 1 per cent human gamma globulin, and 0.15 M NaCl).

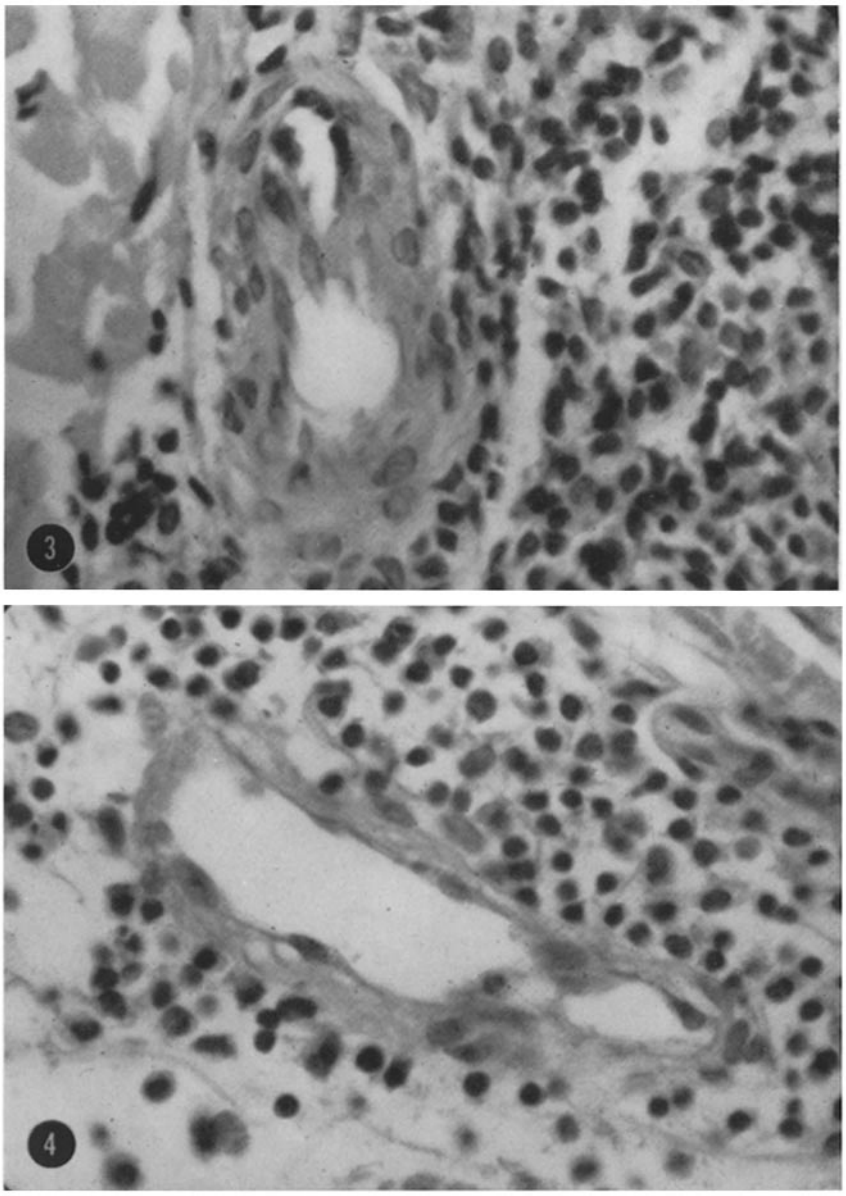


(Maurer: Immunologic studies)

PLATE 106

FIG. 3. Histological section (hematoxylin and eosin) of biopsy of 24 hour skin reaction of sensitized individual. Note almost pure lymphocytic response. × 500.

FIG. 4. Histological section (hematoxylin and eosin) of biopsy of 24 hour skin reaction of normal individual injected with extract of leukocytes from sensitive donor. Note again marked lymphocytic infiltration. × 500.

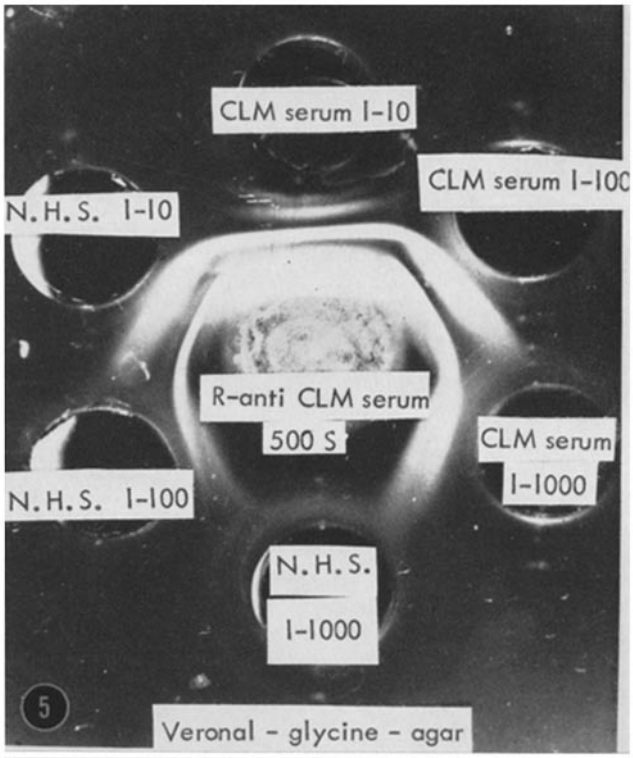


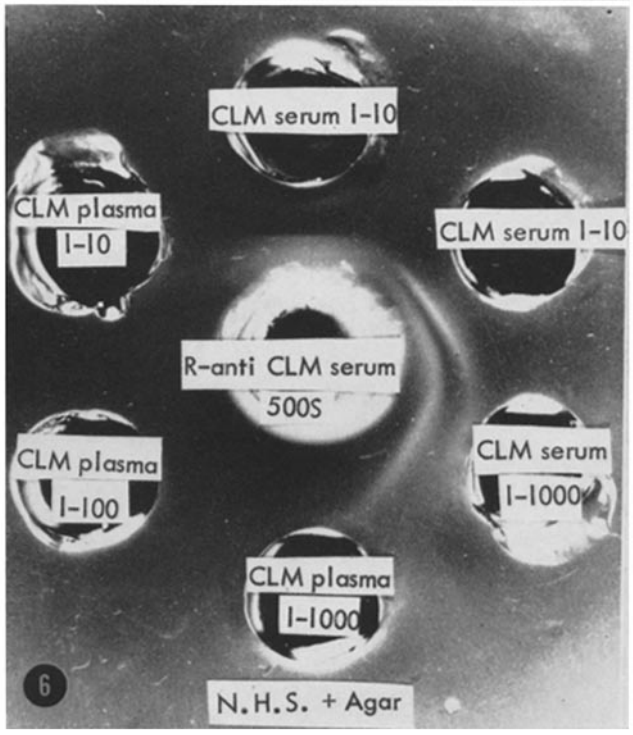
(Maurer: Immunologic studies)

PLATE 107

FIG. 5. Patterns of reaction in agar of CLM serum and normal human serum with rabbit anti-CLM serum 500S.

FIG. 6. Patterns of reactions in medium of normal human serum 25 per cent and agar of CLM serum and anti-CLM serum 500S. Negative reactions noted with a plasma treated with ethylene oxide under mild conditions.





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