adjuvant. (3) Intravenous injection of these antigens did not result in production of CA antibodies in high titer, but O antibody titers were similar to those achieved with adjuvant. (4) Intravenous administration of *S. typhimurium* antigen into animals injected previously with Freund's adjuvant into the footpad, failed to elicit CA antibody production. (5) Feeding of viable *E. coli* 014 led to a moderate CA antibody response. In contrast, feeding of *S. sonnei* or *E. coli* 0111 engendered O antibodies but not CA antibodies.

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Received September 3, 1963. P.S.E.B.M., 1963, v114.

Erythrocyte-Modifying Capacity and Serological Reactivity of Cell Components of Human Mycoplasma (PPLO) Strains. (28776)

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Quantitation of antibody to mycoplasma organisms and determination of serological interrelationships among PPLO (pleuropneumonia-like organisms) have usually been determined with complement-fixation(1-4), agglutination(5-7), or, more recently, with indirect fluorescent antibody(8-10), latex-fixation(11), or gel diffusion(12) procedures. Direct and indirect hemagglutination and hemagglutination-inhibition tests have only been found useful in detecting antigen and estimating antibody levels against pathogenic avian(13,14), bovine(15), and porcine(16) PPLO.

The absence of a rigid cell wall containing polysaccharide or mucopolysaccharide in this group of organisms(17,18) has made the recovery of substances capable of sensitizing erythrocytes more difficult. Most investigations on isolation of serologically active fractions from mycoplasma have been carried out with the agent of contagious bovine pleuropneumonia, *Mycoplasma mycoides* (19-23).

Human strains of mycoplasma have been subjected to fractionation procedures in an effort to identify cell components participating in serological reactions and to determine whether these fractions would sensitize erythrocytes sufficiently to detect PPLO antibody in an indirect hemagglutination procedure.

Materials and methods. PPLO. The following human types and designated strains were used: *M. hominis,* type 1 (PG-25), obtained from J. S. Bailey, George Washington Univ.; *M. hominis,* type 2 (Campo), *M. fermentans,* type 3 (PG-18), *M. salivarium,* type 4 (PG-20), and *M. pneumoniae* (Eaton-FH strain) obtained from Dr. R. M. Chanock, NIAID, NIH; *M. hominis,* type 2 (07) and a saprophytic strain, *M. laidlawii* obtained from Dr. M. F. Barile, Division of Biologic Standards, NIH.

Cultivation. Ten liters of liquid media consisting of 9 parts of Difco PPLO broth, 1 part of 25% yeast extract, and 1% Difco PPLO serum fraction were used for all organisms except the PG-25 and Eaton PPLO. For these 2 strains, 10 liters of media containing 7 parts of broth, 1 part of 25% yeast extract, and 2 parts uninactivated horse

serum (Cappel) were employed. In addition to the above constituents, the final media also contained 1000 units of penicillin and 5 μg of amphotericin per ml and a 1:2000 concentration of thallium acetate. Subcultivations were made every 3-4 days to increasing quantities of media, utilizing approximately 10% inocula, and the final culture maintained for 3 days at 37°C in an atmosphere of 10% CO_2 in air. The Eaton strain was harvested after 10 days under the same growth conditions.

Extraction technic. Liquid cultures were harvested in an RC-2 Servall refrigerated centrifuge with a Szent-Gyorgyi and Blum continuous flow system at 17,000 rpm (34,800 $\times$ *g*). The packed cells were washed once in phosphate-buffered saline (pH 7.0) and packed again at 10,000 rpm for 40 minutes. Cells grown in 20% horse serum were washed twice in buffered saline. Packed cells were extracted in a manner similar to that employed by Yoshida(23). Lipids were removed by 3 extractions with 10 ml each of acetone and 3 extractions with 10 ml each of ether. The supernatants were pooled, dried down under vacuum, and the lipid residue suspended in 10 ml of phosphate-buffered saline. The cells were suspended in 40 ml of 0.5 N NaOH, frozen and thawed 3 times to break up cells, and then held at room temperature for 2 hours. The cell residue was packed at 10,000 rpm for 40 minutes and rehydrolized twice more with 10 ml of 0.5 N NaOH at room temperature for 1 hour. The pooled supernatants were precipitated with 10% trichloroacetic acid and this treatment repeated twice more. Precipitated protein was removed by centrifugation, the remaining supernatant fluid was poured into 4 volumes of ice-cold acetone and allowed to stand at 4°C overnight. Resulting acetone-precipitated material was dissolved in distilled water and re-precipitated twice more in 4 volumes of cold acetone and the final sediment suspended in 20 ml of buffered saline and labeled "polysaccharide fraction." The precipitate recovered from trichloroacetic acid treatment was suspended in 20 ml distilled water, dialyzed against running tap water for 3 days, adjusted to pH 8.5 with 1 N NaOH, and poured into 4 volumes of cold acetone. Following centrifugation at 10,000 rpm for 1 hour, the sediment was resuspended in 20 ml of distilled water at pH 7.4, again sedimented, and the final precipitate suspended in buffered saline and labeled "protein fraction."

Agglutination technic. Whole cell antigens were prepared from broth cultures, as outlined earlier, centrifuged, washed, and resuspended in buffered isotonic saline containing 1:10,000 merthiolate to a 50% light transmittance at 650 mμ in a Model B Beckman spectrophotometer. Hyperimmune rabbit antisera were prepared in a manner similar to that described by Bailey *et al*(7). Two-fold dilutions of antisera were made in 0.3 ml volumes of buffered saline, an equal quantity of antigen added, and tubes incubated at 52°C overnight. Appropriate pre-immunization sera and antigen controls were included in each test.

Fluorescent antibody (FA) technic. Indirect immunofluorescence tests(8) were carried out utilizing either agar plate antigens, transferred to glass slides by the hot water technic of Clark *et al*(24), or washed, preserved, broth antigens similar to those used in agglutination tests. The latter were found to be easier to prepare and gave less nonspecific fluorescence than antigens removed from agar plates. Gentle heat fixation was used for all broth antigens except Laidlaw suspensions where methanol fixation was found necessary for adherence to the slide. Antisera dilutions were made in saline containing 1% normal rabbit serum. Sheep anti-rabbit globulin fluorescein conjugate was obtained from Progressive Laboratories. A fluorescent antibody-inhibition test was used to detect serological activity of isolated PPLO fractions. Small quantities (0.2 ml) of specific PPLO antisera dilutions were placed in tubes and an equal quantity of PPLO antigen fraction added. All tubes were incubated in a 37°C water bath for 10 minutes and one drop of this mixture placed over dried, fixed PPLO broth antigen on slides. Following incubation at room temperature for 30 minutes and washing in buffered saline, one drop of a suitable dilution of fluorescein tagged anti-

TABLE I. Serologic Relationships Among Human Type PPLO Strains as Measured by Agglutination Tests.

PPLO antigen	Agglutinin titers of rabbit antisera to PPLO strains: Type 1 (PG-25)	Type 2 (Campo)	Type 2 (07)	Type 3 (PG-18)	Type 4 (PG-20)	Eaton	Saprophytic (Laidlaw)
PG-25	640*	N†	N	N	20	N	N
Campo	20	320	320	N	N	N	N
07	20	160	640	N	N	N	N
PG-18	N	10	N	1280	N	N	N
PG-20	N	N	N	80	640	N	N
Eaton	N	N	10	20	20	160	N
Laidlaw	N	N	N	N	N	N	320

* Reciprocals. † < 1:10.

TABLE II. Serologic Relationships Among Human Type PPLO Strains as Measured by Indirect Immunofluorescence.

PPLO antigen (agar plate)	Indirect fluorescent antibody titers of rabbit antisera to PPLO strains: PG-25	Campo	07	PG-18	PG-20	Eaton	Laidlaw	Normal rabbit serum
PG-25	320*	40	N†	N	N	N	N	N
Campo	40	320	320	N	N	N	N	N
07	N	160	640	N	N	N	N	N
PG-18	40	40	N	1280	40	N	N	N
PG-20	N	N	N	160	640	N	N	N
Eaton	N	N	N	N	N	640	N	N
Laidlaw	N	160	160	N	N	N	640	N

* Reciprocals. † < 1:40.

rabbit globulin was added and the slide again incubated and washed as before. Controls included saline in place of PPLO fractions and addition of whole cell antigen to PPLO antisera dilutions. All spots of fixed antigen were mounted in 10% buffered glycerol and viewed with a Zeiss fluorescent microscope system, utilizing a dark-field condenser, an Osram HBO 200 high pressure mercury vapor lamp, ultraviolet excitation filters UG-2 and UG-5, and a red barrier filter.

Hemagglutination procedures. Washed 10% suspensions of human type O erythrocytes were sensitized with equal quantities of PPLO antigen fractions and titrated in a manner similar to that described by Landy and Lamb (25) for estimating Vi antibody. For tanned erythrocytes, equal quantities of 2.5% erythrocytes and 1:20,000 tannic acid in isotonic saline were incubated at 37°C for 10 minutes and washed once in buffered saline. Five ml quantities of 2.5% tanned erythrocytes were sensitized with 1.0 ml of PPLO fractions for 30 minutes at 37°C, the cells sedimented, washed once, and resuspended to 1.25% concentration in 1:250 normal rabbit serum-saline. Two-fold dilutions of anti-PPLO rabbit serum were made in 1:100 normal rabbit serum-saline and 0.1 ml of tanned, sensitized erythrocytes added to 0.2 ml of serum dilution. Patterns of sedimented erythrocytes were read after 2 hours at 37°C. Controls included pre-immunization rabbit serum against sensitized erythrocytes, pre- and post-immunization sera against unsensitized erythrocytes, and modified and untreated erythrocytes in diluent.

Results. Agglutination and indirect fluorescent-antibody tests (Tables I and II) generally revealed the clear-cut serologic distinctions among well known PPLO strains isolated from human sources. The type 4 oral strain appeared to share some antigenic properties with the type 3 genital strain in both test procedures. The cross reaction between type 2 human PPLO and the Laidlaw strain observed in the FA test is unexplainable at present and has not, to our knowl-

TABLE III. Hemagglutination Tests with Human Erythrocytes Sensitized with PPLO Cell Fractions.

RBC's sensitized with fraction from PPLO strain:	Cell fraction	Hemagglutinin titers of rabbit antisera to PPLO						
		PG-25	Campo	07	PG-18	PG-20	Eaton	Laidlaw
PG-25	"protein"	120*	15	N†	15	15	7.5	7.5
PG-20	"	15	15	N	30	480	N	N
Eaton	"	N	N	N	N	N	30	N
Laidlaw	"	7.5	N	N	N	N	N	480
	pH 7.4 "protein" wash	N	N	N	N	N	N	1920

* Reciprocals. † < 1:7.5.

edge, been previously reported. Identical FA titers were obtained with new Laidlaw antigen and a second series of rabbits immunized with the Campo strain, while pre-immunization rabbit sera at a dilution of 1:20 was negative to Laidlaw antigen.

Fractionation of washed cells into "lipid," "protein," and "polysaccharide" components resulted in varying amounts of each fraction from different strains. Discernible indirect hemagglutinating activity was not observed with either the "lipid" or "polysaccharide" fractions, as prepared, and neither tanned nor untanned human or sheep erythrocytes appeared to be sensitized by these fractions. However, "protein" fractions from 3 of the strains employed were found to adsorb to tanned human erythrocytes sufficiently for measurement of homologous antibody (Table III). Hemagglutination test titers were lower than agglutination or indirect fluorescent-antibody titers when PPLO "protein" fractions were used for sensitization. In only one instance was the "protein" pH 7.4 wash found active in sensitization of red cells but this activity appeared more consistent with the higher titers usually observed in hemagglutination tests. The sensitization of red cells with fractions recovered from the Eaton agent gave equivocal results. Table IV shows the results of dilution on the ability of 2 of the PPLO fractions to sensitize erythrocytes.

The indirect immunofluorescent-inhibition tests (Table V) indicated a correlation between the fractions active in erythrocyte sensitization and the absorption of PPLO antibody. None of the "polysaccharide" or "lipid" fractions significantly reduced the indirect FA titer of homologous antisera while "protein" fractions decreasing the indirect FA titer of PPLO antibody 8-fold or more were also adsorbed to red cells and hemagglutination observed in the presence of homologous antibody. The "protein" fraction from Eaton agent demonstrated some immunofluorescent-inhibition but, as observed in hemagglutination tests, the results were less than those observed with the other active preparations.

Discussion. A practical, sensitive, and specific serological test is needed for measuring the immunological response of hosts to PPLO and for identifying and classifying serotypes of mycoplasma. While the agglutination test gives clear distinction among laboratory strains of mycoplasma, some difficulty is usually encountered in maintaining antigen stability of freshly-isolated strains from man, especially those recovered from the oral cavity. The indirect immunofluorescent proced-

TABLE IV. Effect of Antigen Dilution on PPLO Hemagglutination Tests with Tanned Human Erythrocytes.

PPLO strain	Cell fraction	Antigen dilution for RBC sensitization	Hemagglutinin titer of homologous rabbit antisera
PG-25	"protein"	undiluted	1:120
		1:4	1:7.5
		1:8	Neg
		1:16	"
Laidlaw	pH 7.4 "protein" wash	undiluted	1:1920
		1:4	1:240
		1:8	1:60
		1:16	1:15
		1:32	Neg

TABLE V. Serological Activity of Cell Fractions from Alkaline Hydrolysis of PPLO in an Indirect Fluorescent-Antibody Inhibition Test.

PPLO antigen (broth)	Indirect FA titers of homologous PPLO rabbit antisera adsorbed with PPLO cell fractions or whole cells				
	Control (saline)	"Polysaccharide"	"Protein"	"Lipid"	Whole cells
PG-25	256*	128	32	128	<4
Campo	256	256	256	256	16
07	512	512	512	512	16
PG-18	512	512	512	512	4
PG-20	512	512	32	256	16
Eaton	256	256	64	128	8
Laidlaw	256	128	32	128	4
"	512	—	32†	—	16

* Reciprocals. † pH 7.4 "protein" wash.

ure seems to be the most suitable test at present. The use of washed and concentrated broth PPLO antigens in this test reduced the nonspecific fluorescence occasionally observed with antigens transferred from agar surfaces.

The results presented here indicate that while fractions capable of sensitizing erythrocytes can be separated from human type mycoplasma, they are recoverable only in small quantities and do not retain their activity over a wide dilution range. Since the quantities of active material were limited, no attempt was made to characterize chemically these fractions although some preliminary tests have shown several of the "polysaccharide" components to give positive Molisch reactions and all of the "protein" fractions to be biuret positive. A direct relationship was observed between the ability of a preparation to adsorb antibody reacting in the immunofluorescent test and its capacity to sensitize erythrocytes. This correlation was evident only with "protein" antigens obtained from PPLO by alkaline hydrolysis and trichloroacetic acid precipitation. Other investigators have shown a relationship between the lipid of *M. mycoides* and complement-fixing antigen(20,23), while erythrocytes sensitized with isolated polysaccharide were more active in reducing agglutinin than complement-fixing titers(15).

Some studies on the erythrocyte-sensitizing properties of alcohol-precipitated(20) and heated PPLO preparations(16) have been carried out in this laboratory. Fractions recovered from ethanol treatment of type 2 mycoplasma, when adsorbed to normal human erythrocytes, gave hemagglutinin titers of 1:960 to 1:1920 against homologous antisera while also decreasing the indirect FA titer of type 2 antisera 8-fold. Alcohol-precipitated fractions from the other PPLO types were not active in either test. Heated (100°C – 10 minutes) suspensions of several PPLO strains (07, PG-18 and Eaton) were also found active in hemagglutination procedures when tanned sheep erythrocytes were employed.

The hemagglutination and FA inhibition tests are now being employed in a study of the immunological characteristics of other PPLO antisera. Additional investigations on the antigenic and endotoxic properties of the isolated mycoplasma fractions are in progress.

Summary. Liquid cultures of several mycoplasma strains were harvested and packed cells subjected to fractionation procedures. Recovery of fractions from alkaline hydrolysis yielded small quantities of material capable of modifying erythrocytes for specific agglutination in homologous PPLO antiserum. Fractions capable of sensitizing erythrocytes were also active in adsorbing specific PPLO antibody reacting in an inmmunofluorescent test. Hemagglutinin titers of PPLO antisera were compared to agglutinin and indirect fluorescent antibody titers.

The author wishes to express his appreciation to Dr. Norman B. McCullough, Chief, Laboratory of Bacterial Diseases, NIAID, NIH, for his support and critical review of the manuscript and to C. Winslow Renshawe for excellent technical assistance.

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Received September 3, 1963. P.S.E.B.M., 1963, v114.

Mammary Carcinoma in Mice Bearing a Transplantable Mammotropic Tumor, Carrying the Bittner Virus.* (28777)

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Induction of transplantable mammotropic pituitary tumors (MtT) in LAF_1 (C57L × A/He) female mice by radiation was reported in 1956(1). Later, the estrogen-induced pituitary tumors were also identified as mammotropic(2). Subsequent studies of numerous MtT induced by radiations and estrogens led to the conclusion that the hormones of MtT alone are not carcinogenic(3). In LAF_1 mice the mammary tumor virus of Bittner (MTV) alone failed to induce mammary tumors (MT) in adult LAF_1 female mice, but MtT rendered such mice susceptible to MTV(3). Similarly MtT renders adult female rats susceptible to induction of MT by subthreshold doses of 3-methylcholanthrene(4) and radiation(5).

Recently an MtT strain, used in co-carcinogenesis studies with MTV(3), appears to have been "contaminated" with MTV so that it now alone produces in adult LAF_1 female mice not only an extraordinary mammary gland hyperplasia with anaplasia but also numerous MT.

Materials and methods. Mice. Virgin female LAF_1 (C57L/J × A/HeJ) mice, about 6 to 8 weeks old, supplied by the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine were fed commercial pellets and tap water *ad libitum*. The incidence of mammary carcinoma in these mice was about 1% (3,6-8).

MtT. The functional MtT strain 38 used arose in a female LAF_1/J mouse that had been given 550 r X-rays over the head and neck and 0.05 mg pellets of diethylstilbestrol (DES) renewed at 3-month intervals(2). First passage tumors of this strain were used

* This investigation was supported by Nat. Cancer Inst.