Isoimmunization with Human γG* Immunoglobulins. (31098)

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Studies in mice(1), rabbits(2), guinea pigs (3), non-human primates(4) and humans(5) have disclosed that a serum protein may exhibit antigenic heterogeneity within a species. Identification of antigenic determinants on proteins permits individuals to be grouped by natural markers. Among the serum proteins bearing these natural markers are the immunoglobulins which have special significance because of their role in immunologic and allergic reactions and in maternal-fetal interactions.

The reagents used for the study of the antigenic specificities of the γG-immunoglobulins in man depend on the availability of serum rheumatoid factors of some rheumatoid arthritic patients or from normal individuals with serum proteins that have the unique and selective ability to agglutinate red blood cells coated with human γG-proteins. The paucity of these reagents and the technical difficulties encountered in their standardization have made it difficult to obtain information concerning the genetics and chemistry of human γG-immunoglobulins. For review see(6).

Direct isoimmunization of mice or rabbits with serum proteins elicits precipitating antibodies suitable for genetic and quantitative immunochemical studies. In earlier work we sought to prepare precipitating antibodies which would identify antigenic differences in individual human proteins by immunizing non-human primates with human serum proteins(7,8). The precipitins revealed heterogeneity among the γG-immunoglobulins, subsequently shown to be related to the heavy chain antigenic determinants on the major subclasses of γG-myeloma proteins(9). However, these antigenic specificities reflect species heterogeneity rather than individual differences in a normal human population.

The object of the present work was to explore the possibilities offered by direct isoimmunization of human volunteers with human γG-immunoglobulins from individual donors and to ascertain whether it was possible to obtain reagents uniquely suited for genetic and immunochemical studies of the human γG-immunoglobulins.

Materials and methods. Healthy laboratory personnel provided serum from which the γG-immunoglobulin antigens were prepared. The serum proteins from an individual donor which precipitated in 1.27 M sodium sulphate were suspended in 5-10 ml of pH 8.6 buffered saline (0.64 g boric acid, 0.97 g sodium tetraborate decahydrate, 9.44 g sodium chloride, water to make 1 liter) and dialyzed 24 hours against 2 changes of 0.01 M phosphate buffer pH 8.6 (9.89 g tris (hydroxymethyl) aminomethane, 0.68 ml 15.7 M phosphoric acid, water to make 1 liter). The protein suspension was applied to a column of DEAE cellulose (2.5 × 33 cm) which had been equilibrated with the 0.01 M phosphate buffer. The proteins which did not adsorb to the ion exchange cellulose appeared in the 0.01 M phosphate eluting buffer front. These were concentrated by ultrafiltration at 4°C, to 60-70 mg/ml. Proteins other than the γG-immunoglobulins could not be detected by agar immunoelectrophoresis (60 μg protein) with a potent horse antiserum to whole human serum(8). The concentrated protein solution (antigen solution) was filtered through a Millipore® disc (0.22 μ pore size) cultured for bacterial sterility and stored at −20°C. For the initial injection, the γG-immunoglobulin solution in 0.01 M phosphate buffer was emulsified with an equal volume of Freund's complete adjuvant (85% Bayol 55,† 15% Arlacel A,‡ 0.1 mg/ml heat-killed *Mycobacterium butyricum* in a Spex Mixer§).

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† Formerly Bayol F, Esso Standard Oil, Linden, N. J.

‡ Mannide mono-oleate, Atlas Chemical Industries, Wilmington, Del.

§ Spex Industries, Scotch Plaines, N. J.

Seven healthy men, age 25-40, were selected as volunteers. In the selection of volunteers, a history of previous or recurrent illnesses which have chronic sequelae, *i.e.*, hepatitis, nephritis, rheumatoid arthritis or other hypersensitivity states, blood transfusions, drug or environmental allergies or a reactive skin test to PPD (intermediate strength) of *Mycobacterium tuberculosis* sufficed to disqualify a candidate. Physical examination, chest X-ray, urinalysis and clinical laboratory blood studies (hemoglobin, hematocrit, WBC and differential, fasting blood sugar, blood urea nitrogen, cholesterol, thymol turbidity, alkaline phosphatase, serum glutamic oxalacetic transaminase, serum glutamic pyruvic transaminase, and bromsulfonphthalien retention) suggested no disease. The possible hazards associated with injection of adjuvant and of the development of circulating antibody to γG-immunoglobulin were discussed on several occasions with each man.

Each volunteer was injected intramuscularly, in each arm, with the antigen emulsified in Freund's complete adjuvant, 6 mg γG-protein/0.2 ml emulsion/injection site. Three men (F.T., B.L., A.R.) received 3 booster injections of 1% alum suspensions, each injection containing 5 mg γG-protein, biweekly, given subcutaneously in each forearm. The titer of antibody activity in these 3 men was very low. In the other 4 subjects (B.D., R.D., J.W., J.G.) the booster injections were given intramuscularly in the anterior thigh muscles of both legs, in Freund's complete adjuvant (6 mg γG-protein/0.2 ml emulsion/site) one month after the initial injection. One month later, a second similar booster injection in Freund's complete adjuvant was given intramuscularly into each arm into areas not previously injected. Thus each of these 4 men received 36 mg γG-protein in 1.2 ml of emulsion divided into 6 sites over a 3-month interval.

Antibody activity was assayed by hemagglutination techniques in which red cells are coated with incomplete Rh antibodies of a known Gm or Inv. type and agglutinated in the presence of human antiserum(5,6). Precipitating antibodies were assayed by the agar diffusion method of Preer(10) and by microquantitative precipitin methods involving protein determination of washed antigen-antibody precipitates from 3.0 ml of antiserum to which increments of homologous antigens were added (5-8 γ N to 36-48 γ N)(11).

TABLE I.* Antibody Response of Human Volunteers Immunized with Human γG-Immunoglobulins in Freund's Complete Adjuvants.

Serum	Gm type a	b	x	f	Inv. type a	Antiserum specificity
Donor E.L.	+	+	0	+	0	
Recipient B.D.	0	+	0	+	aggl.	Gm (a)
" R.D.	+	+	0	0	+	Gm (f)
Donor C.H.	+	+	0	0	+	
Recipient J.W.	0	+	0	+	0	Gm (a)
" J.G.	+	+	0	+	0	Normal serum agglutinator
Donor W.H.	+	+	0	—	+	
Recipient F.T.	+	+	0	+	0	Inv. (a)
" B.L.	+	+	0	—	0	" "
" A.R.	+	+	0	+	0	" "

* Gm typing by Dr. Hugh Fudenberg, Univ. of California Med. Center, San Francisco.

Results. All 7 volunteers developed hemagglutinating antibodies to γG-immunoglobulins coated on red blood cells. Weekly serum samples revealed that hemagglutinating antibodies were present by the 4th or 5th week after immunization. After the 6th or 7th week the titer and antibody specificity were unchanged over the subsequent 20 months of observation. The reciprocal dilution titer of antibody in the 3 men receiving alum precipitate boosters was 1:2-1:4. The antibody titer in the 4 men receiving boosters in Freund's adjuvant ranged from 1:16-1:128.

Table I shows the immunologic results of isoimmunization of human volunteers with γG-immunoglobulins from single donors. The Gm and Inv. types of the serum γG-immunoglobulins of each donor and the recipients are shown on the left. The column on the right shows the specificity of the antiserum. Recipient B.D. lacked the Gm (a) specificity present in the donor E.L. and responded to immunization by raising antibodies directed against Gm (a) determinants. Although recipient R.D. differed from donor E.L. in Gm (f) and Inv. (a) specificity, his antiserum reacted with Gm (f) proteins but it did not react with Inv. (a) antigens. The re-

sponse was selectively against the Gm (f) determinants. In the set of immunizations with donor C.H., the recipient J.W. lacked both Gm (a) and Inv. (a) specificity but had Gm (f) in his serum; antiserum J.W. detected only Gm (a) antigenic determinants. The recipient J.G. lacked Inv. (a) determinants; his pre-injection serum sample reacted with all normal sera tested. The hemagglutination antibody titer remained unchanged after immunization. Cells coated with Inv. (a) type light chains were not available to test for this antibody specificity. In the third set of immunizations the serum of donor W.H. was Inv. (a) positive and each of the recipients were Inv. (a) negative. They responded to immunization with antisera directed against the Inv. (a) specificity.

Precipitating antibodies were not demonstrable, even with the sensitive agar diffusion method of Preer and of micro-quantitative precipitation.

Several serum samples from each volunteer, taken at various intervals after immunization, were examined for the possible development of antinuclear and antimuscle antibodies by immunofluorescence methods; none were found.‖

Tests for hemagglutinins were negative for cold and warm saline factors against normal human red cells as well as by Coomb's Test with incomplete antibodies of Rh, Kell and Fy^a factors.¶ These incomplete antibodies presumably lacked the antigenic determinants which our human antisera were able to detect when the cells were coated by selected incomplete Rh antibodies and hemagglutination was demonstrated.

Immunoelectrophoresis of volunteer sera during the course of the study showed unchanging normal patterns of the immunoglobulins with both horse and monkey antisera to the human immunoglobulins(8). As judged by paper electrophoresis, total serum proteins and their relative proportions also remained unchanged.

The response to the injection of Freund's complete adjuvant in the volunteers merits some comment. Inflammation and tenderness appeared at the injection site 24 to 48 hours after giving the emulsion. This was associated with mild symptoms of malaise, nausea and 2-3°F fever. These symptoms and much of the tenderness abated in 48-72 hours with full recovery in 7-10 days. In the men receiving alum suspensions, the site of this injection was erythematous and edematous for 3-4 days with recovery in 7-10 days. No systemic symptoms or activation of the previous emulsion sites were noted. In the 4 men who received emulsion boosters, the response was similar to that with the first injection, *i.e.*, nausea, malaise and fever appearing in 48 hours at the time of marked tenderness and inflammation. These symptoms and signs of inflammation abated in 48-72 hours with clinical recovery in 7-10 days. Earlier sites of emulsion injections were not clinically reactivated. This clinical response is reminiscent of administration of *Salmonella typhosa* or other injections with endotoxic products.

The 3 men who received alum boosters (F.T., B.L., A.R.) and one of the men who received emulsion boosters (J.G.) have had no other sequelae. One (R.D.) developed a sterile abscess in one arm 6 months after immunization which was treated by surgical excision; another man (J.W.) developed a sterile abscess in each arm, 6-7 months after immunization, at the sites of the first injections, which healed completely after surgical excision, and the third man (B.D.) developed a sterile abscess in each arm, 6-7 months after immunization, at the sites of the second booster injections. Two excision procedures of each abscess were followed by healing and recovery. No other evidence of clinical or systemic disorders have appeared in any of the men in the course of 24 months of observation.

Histologic examination of the excised tissues disclosed chronic inflammatory changes. In volunteer B.D., who required reexcision, a characteristic talc granule was identified in one field by polarized light and a tuberculoid granuloma was seen in another although acid-fast bacteria could not be demonstrated.

The skin test to P.P.D. (intermediate)

‖ Courtesy of Dr. Arthur Strauss, Laboratory of Immunology, NIAID, Nat. Inst. Health.

¶ Courtesy of Dr. Paul Holland, Clinical Center Blood Bank, Nat. Inst. Health.

from *M. tuberculosis* remained negative in the volunteers for 9 months but not one year after the immunizations (interval of skin testing). The positive response measured 10-13 mm induration at the time of conversion whereas 3 mm induration or less was observed before immunization in all the volunteers. This conversion response represents reactivity to cross reacting antigens since the volunteers received *M. butyricum* in the immunizing emulsion.

Discussion. Human volunteers have been immunized with individual human γG-immunoglobulins emulsified in Freund's complete adjuvant. The resulting antisera were found to contain hemagglutinating antibodies specific against the Gm determinants of the donor's protein. Other reports of the antigenicity of the Gm determinants appear to result from indirect or fortuitous immunizations associated with therapeutic or natural events, *i.e.*, multiple blood transfusions(12), injections of pooled Cohn fraction II(13) and transplacental transfers during a normal pregnancy(14,15). In the present report, unequivocal evidence has been provided for the antigenicity of Gm and Inv. determinants in normal individuals. When the donor and recipient differed in both Gm and Inv. determinants (E.L. *vs* R.D., C.H. *vs* J.W.), the antibody response was directed against the Gm determinant. Since the Inv. (a) specificity is an effective antigen (W.H. *vs* F.T., B.L., A.R.) the selective response to the Gm determinants, when the recipients differ in both Gm and Inv. type, suggests the existence of a competitive antigen situation. The presence of a Gm (f) determinant in the recipient but absent in the donor did not appear to be inhibitory to the antigenic stimulus.

The high degree of specificity of antibodies induced by isoimmunization may eventually lead to identification of antigenic subgroups of the γG-proteins. Recognition and structural localization of antigenic subgroups should provide markers useful for elucidation of genetic mechanisms which control synthesis of these peptides and for clarifying the nature and source of normal serum agglutinating antibodies, rheumatoid factors and the absence of Gm antigenic specificities on some myeloma proteins but not others.

Although precipitating iso-antibodies can be induced in some animals with particles or cells coated with γG-protein, our best results have been obtained with subcutaneous or intramuscular injections of protein antigens in Freund's complete adjuvant. Booster injections of particulate antigen were ineffective in producing precipitins for some selected rabbit allotypes; it was necessary to subject these rabbits to repeated booster injections in Freund's incomplete adjuvant for as long as 2 years. In our experience, immunization of monkeys with protein antigens in alum precipitates or emulsions with Freund's incomplete adjuvant was ineffective in eliciting precipitins to human γG-proteins. On the other hand, protein antigens emulsified with Freund's complete adjuvant evoked excellent titers of precipitins in monkeys(7, 8). Five chimpanzees injected with human γG-immunoglobulins in Freund's incomplete adjuvant followed by both Freund's incomplete adjuvant and alum boosters did not develop precipitins. When γG-proteins in Freund's complete adjuvant were given to 5 other chimpanzees and one orangutan,** precipitating antibody was demonstrable in 3 of the chimpanzees. The immunizing and booster injections in complete or incomplete adjuvant were not followed by abscess formation. Three of these animals had unrelated neurosurgical procedures several months or years after immunization. They received blood transfusions of human or chimpanzee origin without evidence of transfusion or other immunologic reactions, although precipitating and non-precipitating antibodies could be demonstrated in the animals at the time of surgery. In other studies, monkeys sacrificed 2 to 3 years after immunization still retained emulsion at some injection sites and, except for histologic evidence of chronic inflammation in the area of the injection site, no pathologic changes were seen.

Since γG-immunoglobulins given in Freund's incomplete adjuvant or in the form of alum precipitated antigen have consistently failed to produce precipitating isoantibodies in our laboratory in non-human pri-

** Courtesy of Dr. Jan Moor-Jankowski, New York Univ. School of Med.

mates, it was felt that for development of precipitating antibodies the greater immunizing potential of Freund's complete adjuvant was an essential requirement. In the light of our overall experience, it seemed a reasonable expectation that prolonged immunization with γG-immunoglobulins in Freund's incomplete adjuvant would be ineffective in human subjects as well. Based on the aforegoing, and with due consideration of possible hazards, it was elected to use Freund's complete adjuvant as the vehicle for human isoimmunization. To minimize possible untoward reactions from the mycobacterial component, only tuberculin negative subjects were selected, and the source of the microbial component was restricted to *Mycobacterium butyricum*.

While Freund's adjuvant has been used extensively in animal experiments, there remains a paucity of data on its use and particularly on its effects in man. Ragweed antigens emulsified in mineral oil (Freund's incomplete adjuvant), or emulsified mineral oil alone have been given to a total of 1768 ragweed sensitive patients once or twice during the pollen season for one to six years. There has been no evidence of systemic disease attributable to the adjuvant injections(17,18). In connection with field trials of viral vaccines, mineral oil emulsion (Freund's incomplete adjuvant) has been administered to more than 45,000 patients; now, 10-15 years later, no generalized or systemic complications attributable to the adjuvant have been reported in this large population(19-22).

Our experience with 7 volunteers does not warrant extensive clinical generalizations although these volunteers have now been observed for 24 months. Despite the development of, and continued presence of, circulating antibodies to γG-immunoglobulins, clinical and laboratory examinations have revealed no evidence of generalized systemic or immunologic disorders. These findings are in agreement with reports that the presence of circulating anti-γ-globulin factors in normal individuals(14,15), patients and their families with rheumatoid factors, or patients who have received multiple transfusions, have not been causally related to disease(23) even though the possibility exists for transfusion reactions following infusion of large amounts of plasma with a Gm-type incompatibility.

On the basis of detailed clinical and immunological observation of this group of 7 volunteers over a period of 2 years the following comments seem warranted: 1) In every instance the subjects developed isoantibodies. 2) Untoward reactions and complications have been minimal. 3) Despite the effectiveness of Freund's complete adjuvant in evoking isoantibodies in man, the occurrence of local reactions and the possibility, albeit remote, of some form of generalized immunologic disease or disseminated granulomata would militate against the use of Freund's complete adjuvant in man as a method of choice for routine immunizations.

Summary. 1. Human volunteers immunized with human γG-immunoglobulins emulsified in Freund's complete adjuvant have responded by producing non-precipitating isoantibodies to γG-immunoglobulins. 2. These iso-antibodies, uniquely specific against Gm and Inv. antigens, were demonstrated by sensitive hemagglutination methods. 3. During 2 years observation of 7 volunteers, no evidence of immunologic disease was discerned. 4. Local reactions were such as to deter use of Freund's complete adjuvant in human immunization.

Antigen typing and antibody specificity were determined by Dr. Hugh Fudenberg, Univ. of California, Med. Center, San Francisco. Laboratory determinations were carefully performed by the Clinical Laboratory, U. S. Penitentiary, Lewisburg, Pa. The cooperation of Mr. Olin Blackwell, Warden and the Medical Staff of the U. S. Penitentiary, Lewisburg, Pa. is gratefully acknowledged.

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Chromosome Studies of Human Cells Infected *in utero* and *in vitro* With Rubella Virus.* (31099)

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Published observations on the induction of chromosome breakage by a number of viruses such as measles, chickenpox and herpes simplex(15,1,2,7,8) as well as the recent occurrence of a major epidemic of rubella in the United States have stimulated interest in the possible effects of rubella on the chromosomes of human fetuses *in utero*. In fact, positive effects of rubella virus on chromosomes *in vivo* have been reported by Wiedemann(25) who found elevated breakage in 2 cases of Gregg's syndrome.

Further, it was demonstrated in our laboratory that infection of human fibroblasts *in vitro* resulted in an increased frequency of breakage, although neither major rearrangement nor evidence of non-disjunction was observed(4). On the basis of sex chromatin studies it has recently been suggested that a correlation may exist(20) between non-disjunction and epidemic rubella.

On the other hand, no aberrations were observed(13,3) in 2 chromosomal studies of leucocytes from abnormal newborn infants, known to be excreting rubella virus. Earlier, it was reported that no abnormalities were present in cells cultured from fetuses from rubella-infected mothers; however, since only "eight cells" were examined in one case(14) and "mitoses were few" in the other(23) further studies seemed warranted.

In the present study, fibroblast-like cell strains were derived from fetuses which had been aborted therapeutically because of confirmed diagnoses of maternal rubella. In all cases rubella had occurred in the first trimester of pregnancy, usually (60%) in the second month. Further, strains derived from

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