tested in the thromboplastin generation test, it was completely inert in all concentrations studied. It must also be pointed out that the exact relationship of plasmalogens to blood coagulation has not been clarified.

The results of GLC analyses of fatty acid composition of red cell phosphatides recently made by Farquhar *et al.* show an overall similarity to the patterns of platelets.§ This is of importance because it has been found that red cell phospholipids can promote formation of blood thromboplastin(14,15).

*Summary.* The fatty acids of blood platelet phosphatides have ben studied by silicic acid, and gas-liquid chromatography. PE-PS fractions contain primarily arachidonic, stearic and oleic acids. Lecithin-sphingomyelin eluates contain principally palmitic, stearic and oleic acids. These components may be quantitatively related to the specialized function of platelets in blood coagulation.

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§ Personal communication.

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## Serologic Studies of Rabbit Antibodies to Rabbit Submaxillary Glands.* (26664)

E. H. BEUTNER, A. Y. DJANIAN, R. C. GECKLER AND E. WITEBSKY
*Department of Bacteriology and Immunology, University of Buffalo School of Medicine and Dentistry, Buffalo, N. Y.*

Experimental evidence has accumulated during the last few years in reference to the pathogenesis of chronic non-specific thyroiditis(1). It has been shown that experimental animals such as rabbits, dogs and guinea pigs when immunized with extracts of thyroid glands of the respective species frequently develop thyroid specific antibodies demonstrable either by the usual serological tests as circulating antibodies or by technics employed to demonstrate delayed hypersensitivity, or both(2). They also frequently show histological evidence of thyroiditis.

Recently attention has been drawn to the pathogenesis of Sjögren's syndrome, a clinical triad characterized by keratoconjunctivitis sicca, xerostomia and also rheumatoid

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arthritis(3,4,5). Some of the same clinical characteristics have been reported in some patients with lupus erythematosus and scleroderma. Bloch and associates(5) have reported results of different serological tests with sera of patients suffering from Sjögren's syndrome, and have referred to the pathology of the salivary gland. Extensive accumulation of lymphocytes and plasma cells was observed in biopsy material obtained from the parotid or submaxillary glands of 10 patients. Experiments have been carried out in our laboratory to determine whether it would be possible to find an organ-specific antigen in the submaxillary and related glands of rabbits; whether that antigen would prove to be antigenic for rabbits and finally, whether lesions would occur in the glands of experimental rabbits which would resemble those seen in Sjögren's disease.

*Materials and methods.* The submaxillary gland of normal rabbits was removed and immediately frozen in liquid nitrogen. Suspensions for immunization purposes were prepared by grinding 1 part of submaxillary gland and 4 parts of buffered saline in a Waring blendor. After blending for about 60 seconds the emulsified material was passed through a plastic screen and stored in the freezer until ready for use. Buffered saline extracts were prepared by centrifuging 10% suspensions of rabbit organs at about 20,000 g for 10 minutes. These suspensions were prepared by homogenizing 1 part of rabbit organ with 9 parts of buffered saline in a Waring blendor. For preparation of smaller amounts of suspension and extract, a glass hand homogenizer was used. Extracts were kept in a deep freeze until ready for use.

New Zealand white rabbits were injected intradermally in the 4 footpads and in several sites in the neck region with a total of 1 cc of a mixture containing equal parts of 20% suspension of rabbit submaxillary gland and complete Freund adjuvant. Mycobacteria used in the adjuvants were prepared according to the method of Colover(6): virulent human tubercle bacilli (strain H37 Rv) are heat killed at 100°C for 30 minutes and extracted in a Soxhlet, first with acetone and then with isopropyl ether. The dried residue is incorporated into incomplete Freund adjuvant, 5 mg per 10 ml. There were 2 groups of rabbits. The first group of 4 rabbits received 2 sets of injections at an 8 week interval. The serological results reported here were obtained with terminal bleedings taken 2 weeks after second injection. A second group of 3 rabbits received a single set of intradermal injections and were bled 3 weeks afterward.

*Complement fixation* was carried out by mixing 0.1 ml of antiserum dilution, 0.1 ml of antigen dilution, and 0.1 ml of guinea pig complement (diluted 1:16 or 1:18). The mixtures were kept for 18 hours in the refrigerator at 4°C, then for 1 hour at 37°C. 0.2 ml of sheep cells sensitized with appropriate dilution of amboceptor was added. Several readings were made, first after the controls had cleared (usually after 15-20 min) and final readings 40-55 min later. Only complete inhibition of hemolysis after clearing of controls was considered as positive.

*The tanned cell hemagglutination test* was carried out using essentially the technic employed in the thyroglobulin studies reported previously(7). The procedure in the present study was as follows: 1) washing human group O Rh positive red cells with pH 7.2 phosphate buffered saline 4 times, 2) tanning cells by mixing a 4% suspension with an equal volume of 1:30,000 tannic acid for 30 minutes, 3) washing 4 times with buffered saline, 4) coating with rabbit submaxillary globulins kept in a boiling water bath for 4 minutes; dilutions of the globulin ranged from 1:50 to 1:1,000, 5) washing 3 times with a 1/100 buffered saline dilution of normal rabbit serum. Antiserum dilutions were also prepared with 1/100 buffered saline diluted normal rabbit serum. Tests were carried out by mixing 0.1 ml of 1.5% coated cell suspensions with 0.1 ml of antiserum dilutions. Readings were based upon observations of the agglutination pattern after standing at room temperature for several hours, refrigerating overnight, and standing for 3 hours. Some of the positive submaxillary antisera exhibited a certain degree of agglutination in dilutions of 1:10 to 1:20 when the tubes were shaken at the end of ob-

TABLE I. Specificity of Rabbit Submaxillary Gland Antisera Tested against Extracts of Organs of Rabbit #1565.

| Antisera, 1:15 dilution | Last extract dilution giving positive reaction | | | | | |
|---|---|---|---|---|---|---|
| | Submaxillary | Parotid | Adrenal | Harderian | Liver | Kidney |
| #1565 | >2700 | >2700 | 30 | <30 | 30 | 100 |
| #1570 | 900 | <30 | 30 | 30 | 30 | 30 |
| #1001 control* | 30 | <30 | 30 | 30 | 100 | 30 |

* Treated with 2 series of injections of complete Freund adjuvant only.

servation time. (The tanned, but uncoated cells were negative). Agglutination readings summarized in Table III represent *patterns* of sedimentation rather than clumping agglutination observed on shaking.

The insoluble material mentioned in Table II was prepared by first making a 10% suspension in buffered saline, centrifuging at about 30,000 g for 10 minutes removing the supernatant extract and resuspending the sediment in 0.25 M sucrose. The suspension was then subjected to differential centrifugation according to the method of Hogeboom and Schneider(8) for isolation of nuclear, mitochondrial and microsomal fractions. The water-soluble extracts were divided into globulins and albumins by precipitation with 50% saturated ammonium sulfate. The washed globulin precipitates were reconstituted to the volume of the original 10% organ extracts by dissolving in phosphate buffered saline, used throughout these studies, made up of 0.115 N sodium chloride and an 0.035 N mixture of sodium and potassium phosphate adjusted to pH 7.2.

*Results.* The antisera obtained by immunization of rabbits with suspensions of pooled freshly frozen submaxillary glands incorporated into Freund adjuvant were examined for presence of circulating antibodies by several serological technics. There was no basic difference in the results between the 2 series, one of which had received one injection, the other series, two injections. Three out of 7 animals revealed the presence of antibodies by means of complement fixation and 6 in the hemagglutination test. The 3 antisera containing antibodies against saline extracts of pooled rabbit submaxillary gland failed to react significantly with saline extracts of other organs. The question arose whether the antibodies produced by isoimmunization would also act on extracts of glands of the antibody-producing animal. A typical experiment is shown in Table I.

Rabbit antiserum #1565 when tested against organ extracts obtained from the same animal fixed complement with extracts of the submaxillary gland and the parotid gland but not with extracts of 4 other organs (Table I). Antiserum #1570 reacted exclusively with the extract of the submaxillary gland but not with the parotid extract nor with the other organ extracs of animal #1565. Aniserum #1001 used as a control shows that the extracts of the submaxillary glands do not fix complement *per se* when tested with rabbit sera. Therefore, the antibodies produced by isoimmunization of rabbits with extracts of the submaxillary gland are of considerable specificity. An additional antiserum #1564 exhibited identical reactivity to that of #1570; namely, it reacted with submaxillary gland but not with other organ extract. In other tests, no reactivity was demonstrable with lung extracts. In tests with extracts of rabbit, cat, and human submaxillary extracts these sera (1565 and 1570) exhibited significant reactivity only with the homologous (rabbit) extract.

Information on the nature of the antigen involved was sought by testing various fractions of the submaxillary glands. Table II summarizes the complement fixing activity of 2 of the antisera, #1565 and #1570, with

TABLE II. Complement Fixation Tests with Submaxillary Gland Components and Rabbit Antibodies to Rabbit Submaxillary Gland.

| Antigens (components of rabbit submaxillary) | Percentage of reactivity of 10% extract | |
|---|---|---|
| | 1565 | 1570 |
| 10% extract | 100 | 100 |
| Globulins | 100 | 100 |
| Albumins | <1 | <1 |
| Microsomes | 25 | 25 |
| Mitochondria | <10 | <10 |
| Nuclei | <10 | <10 |

TABLE III. Tanned Cell Hemagglutination with Rabbit Submaxillary Gland Globulin and Rabbit Thyroglobulin Coated Cells.

| Serum dilutions | NRS | Rabbit sera 1214 (F.A.) | 1565 (R.Sm.) | 1570 (R.Sm.) | 1364 (R.Th.) | 1365 (R.Th.) |
|---|---|---|---|---|---|---|
| | | | Rabbit submaxillary globulin coated cells | | | |
| 1:10 | — | — | ++++ | ++++ | — | — |
| 1:50 | — | — | ++++ | +++ | — | — |
| 1:250 | — | — | +++ | + | — | — |
| 1:1250 | — | — | + | — | — | — |
| 1:6250 | — | — | ± | — | — | — |
| 1:31 × $10^3$ | — | — | — | — | — | — |
| 1:10 u.c. | — | — | — | — | — | — |
| | | | Rabbit thyroglobulin coated cells | | | |
| 1:10 | — | — | — | — | ++++ | ++++ |
| 1:50 | — | — | — | — | ++++ | ++++ |
| 1:250 | — | — | — | — | +++ | ++++ |
| 1:1250 | — | — | — | — | +++ | + |
| 1:6250 | — | — | — | — | ++ | ? |
| 1:31 × $10^3$ | — | — | — | — | — | — |
| 1:10 u.c. | — | — | — | — | — | — |

NRS = Normal rabbit serum.
1214 = Rabbit antiserum to Freund adjuvant emulsified in buffered saline, 3 groups of injections.
1565, 1570 = Rabbit submaxillary gland antibodies, see Table I.
1364, 1365 = Rabbit thyroglobulin antibodies.

the microsomal, mitochondrial and nuclear fractions of the insoluble components of submaxillary suspensions as well as the albumin and globulin fractions of the soluble part.

Fractions of insoluble components were tested at and beyond the limits of the anticomplementary activity. Results were calculated back to the volume of the original 10% suspension (in 0.25M sucrose) for comparison with activity of the 10% extract. Fractions of soluble components prepared by 50% saturated ammonium sulfate precipitation of globulins were adjusted to the volume of the original 10% buffered saline extract. Relative reactivity of submaxillary gland components in terms of reactivity of the crude extract is shown in Table II.

Virtually all of the complement fixing activity appears to reside in the globulin fraction of the extract. Some reactivity was demonstrable in the microsomal fraction, which may be explained by the fact that protein synthesis is considered to be carried out primarily by the microsomal components. Antigenic activity of the microsomes is not included with the activity of the extract because the microsomes are probably not included in the extract as they were very likely sedimented in its preparation. Thus, the sum total of the antigenic activities of the submaxillary gland components appears to be greater than 100%.

Table III summarizes results obtained when rabbit submaxillary antisera #1565 and #1570 were examined for tanned cell hemagglutination activity. For that purpose tanned Rh positive cells of Group O were coated with isolated globulin fraction of rabbit submaxillary gland. The specificity of the reatcion is demonstrated by simultaneous testing with tanned cells coated with isolated rabbit thyroglobulin.

The 2 submaxillary gland rabbit antisera #1565 and #1570 agglutinated tanned cells coated with globulin prepared from pooled rabbit submaxillary gland, but failed to agglutinate tanned cells coated with thyroglobulin (Table III). On the other hand, 2 rabbit antisera immunized with rabbit thyroglobulin agglutinated tanned cells coated with thyroglobulin but not with the globulin of the submaxillary gland. The specificity of the reaction was further examined by means of inhibition of the tanned cell hemagglutina-

tion test. For that purpose antiserum #1565 was mixed with varying dilutions of organ extracts, incubated for 1 hour at room temperature, then tested for its activity in the tanned cell agglutination test. The agglutinating capacity of this antiserum was inhibited by both the rabbit submaxillary extract and the rabbit parotid extract but not by the extract of the rabbit lung or rabbit thyroid. In similar inhibition experiments carried out with the 2 other antisera, #1564 and #1570, the submaxillary extracts exclusively inhibited the agglutination. These results confirm the observations on the spectrum of specificity of these 3 antisera recorded in Table I. Extracts of the submaxillary glands of the antibody producing rabbit inhibited hemagglutination as well as did submaxillary gland extracts of other rabbits.

*Discussion.* From previous investigations it was learned that there are at least 2 methods for considering the presence of experimentally-produced autoantibodies: (1) by immunizing thyroidectomized rabbits with extracts of the rabbit's own thyroid gland, and (2) by isoimmunization of a normal rabbit using thyroid extracts prepared from pooled thyroid glands of different rabbits. In the latter case antibodies obtained should react to the same extent with extracts of all individual rabbit thyroid glands examined, including that of the antibody-producing animal itself. Also, histological changes might be found in the thyroid gland of the antibody-producing animal which then could be considered as an effect of active immunization, (but by no means necessarily an effect of the circulating type of antibodies). In the experiments reported herein evidence for the appearance of antibodies against constituents of the salivary glands of rabbits is presented. So far, histological changes are not demonstrable to our satisfaction in the submaxillary glands of the antibody-producing animal. However, as in the field of autosensitization, histological changes sometimes appear only after a prolonged period of immunization and frequently are preceded by appearance of circulating antibodies. Therefore, this is being further investigated.

These observations might be of interest in human diseases of salivary glands. Urbain and Caroli(4) and others(5) suggest an "autoimmune" etiology of Sjögren's syndrome and related diseases of the salivary and lacrimal glands. Jones(3) reported demonstration of antibodies in sera of patients exhibiting Sjögren's syndrome which precipitate salivary gland extracts. Similar reactions, however, were demonstrated with kidney extracts but not with liver or muscle extracts. More recently, Bloch *et al.*(5) reported briefly on complement fixation tests carried out by Zvaifler and Bloch with Sjögren's syndrome sera and human organ suspensions in which complement was fixed not only in the presence of submaxillary gland suspensions but also with liver, kidney, or striated muscle suspensions. In view of the common association of nuclear autoantibodies with Sjögren's syndrome(5), it seems possible that the observed lack of specificity may be associated with antibodies fixing complement in presence of nuclear antigens. We are now studying the problem whether circulating antibodies specific for the glands involved in Sjögren's syndrome are demonstrable in human sera in addition to non-organ specific antibodies such as nuclear antibodies.

*Summary.* Sera of rabbits immunized with pooled rabbit submaxillary gland suspensions were studied by complement fixation and tanned cell hemagglutination technics. 1. Some of these antisera (3 out of 7) fixed complement in presence of rabbit submaxillary gland extracts. 2. Complement fixing activity of 2 of the 3 sera studied in detail was specific for submaxillary gland extracts within the limits of antigens tested. One of 3 serologically active sera (#1565) cross reacted with parotid gland extracts. 3. The globulin fraction of submaxillary gland extracts contained virtually all the material in the extract which was reactive with rabbit submaxillary gland antibodies. Some activity was also found in the microsome fraction. 4. Extracts of the submaxillary glands of the antibody-producing animals themselves reacted just as strongly as those of other rabbits, indicating the autoantibody nature of the antibodies elicited by isoimmunization.

5. Most of the rabbit antisera (6 out of 7) agglutinated tanned red cells coated with rabbit submaxillary globulin. 6. By hemagglutination inhibition tests, one antiserum (#1565) was found to be inhibited by submaxillary and parotid extracts. The other antisera tested were specific for submaxillary gland antigens. These results agree with those obtained by complement fixation.

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## Production of Glucagon Antibodies and their Role in Metabolism and Immunoassay of Glucagon.* (26665)

G. M. GRODSKY, T. HAYASHIDA,† C. T. PENG AND I. I. GESCHWIND

*Dept. of Biochemistry, Metabolic Unit for Research in Arthritis and Allied Diseases, Dept. of Anatomy and Radioactivity Research Center, School of Medicine, San Francisco, and Dept. of Animal Husbandry, University of California, Davis*

Lack of specificity is a significant disadvantage of certain technics for bioassay of glucagon(1-4). Recently developed methods for immunochemical assay of insulin(5,6) appeared adaptable to the specific measurement of glucagon. This report describes the development of antibodies to glucagon in rabbits treated during a 22-month period, the influence of the antibodies on the metabolism of glucagon and their potential use for immunochemical assay of this hormone. Recently, an independent study of similar nature was reported by Unger *et al.*(7).

*Methods.* Recrystallized porcine glucagon (Eli Lilly & Co., Lot No. 258-234B-93) was used. This preparation contained no detectable amount of insulin when measured by an immunochemical assay described previously(5). Glucagon-$I^{131}$ was prepared by a method used for labeling of insulin-$I^{131}$(8). Specific activity of the preparations varied from 2.5 to 12.0 mc/mg.

Two of 4 male albino rabbits, weighing 2.5-3.0 kg, were immunized with glucagon suspended in either Bayol-Arlacel or Freund's adjuvants, as described by Hayashida and Li(9). The other 2 animals were immunized with glucagon conjugated to ovalbumin with bis-diazo benzidine(10), similarly suspended in the adjuvants. All 4 animals were given subcutaneous injection of the hormone in doses of 3-10 mg at varying intervals over a 2-year period. Serum samples were obtained 2-3 weeks after each series of injections, frozen and stored.

Titers for glucagon antibodies in serum were determined by a modification of the method previously employed for measuring insulin antibodies(5). Glucagon-$I^{131}$, 0.02 $\mu$g, was incubated for 1 hr at room temperature with 0.2 ml of undiluted serum or of serum serially diluted with 5% human serum albumin. Aliquots, 0.1 ml, were subjected to hydrodynamic flow on paper in veronal buffer, pH 8.6, for 1 hr at room temperature. In this system free glucagon remains at the origin, while antibody-bound glucagon travels as a single band with the serum proteins (11). After the papers had been dried and

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† Senior Research Fellow, U.S.P.H.S.