Studies on Immune Responses of Rabbits to Rabbit Submaxillary Antigens.* (30000)

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Rabbits immunized with rabbit submaxillary† gland suspensions in complete Freund adjuvant developed organ specific antibodies to submaxillary extracts(1). Complement fixation tests yielded some cross reactions with parotid gland antigens but not with other tissues tested. Complement fixation and hemagglutination reactions occurred with extracts of the submaxillary glands of the antibody producing animals themselves, thus pointing to their autoantibody nature. The antigenic activity appeared in the fraction of the extract which was precipitable with 2 M ammonium sulfate as demonstrated by both complement fixation and tanned cell agglutination tests.

The present investigation deals with 1) the immune response of rabbits to the ammonium sulfate precipitable fraction of rabbit submaxillary extracts, 2) the presence of precipitating antibodies, and 3) cutaneous hypersensitivity reactions to submaxillary globulins and with 4) the auto-, iso-, and heteroantibodies formed in response to such immunization.

Methods. Antigens. Extracts were prepared from fresh or fresh frozen tissue by grinding 1 part of organ in 9 parts of buffered saline (pH 7.2, 0.115 N NaCl, 0.035 N PO_4) in a Waring blendor for one minute and centrifuging at 20,000 $\times$ *g* in an angle head refrigerated centrifuge for 10 minutes. Globulin fractions were prepared by adding 4.0 M ammonium sulfate in a 1 : 1 (v/v) ratio to the tissue extracts, washing the precipitate in 2.0 M ammonium sulfate and redissolving it in buffered saline.

Immunization. Antisera were prepared in New Zealand White rabbits by injection of the pooled submaxillary gland preparations in equal volumes of incomplete Freund adjuvant with 1 mg/ml Colover's(2) Mycobacterium preparation. The rabbits received a total of 1 ml of the emulsion distributed as 4 footpad injections of 0.1 ml each and several intradermal injections in the neck region. Maximum antibody responses usually appeared in 21 to 35 days after one injection of 2.5 mg of antigen. The antigens or antisera were concentrated by pervaporation or dialysis against sucrose crystals followed by dialysis against isotonic buffered saline.

Immunologic tests. Ouchterlony tests(3) were performed with 1.2% agar‡ containing 1:20,000 merthiolate. Both microwells (2 mm) and macrowells (6 mm) yielded satisfactory bands of precipitation under standardized conditions. The plates were kept at 4.0°C for 2-5 days before filling the wells with reagent to reduce the amount of water expressed into the wells. The precipitation patterns were read every day for 10 days during which time the plates were kept at approximately 26°C. Complement fixation tests were performed as described previously (1). Skin tests were carried out as described in the text.

Immunoelectrophoresis(4). A solution of 1% Noble Agar in 0.02 ionic strength barbital buffer, at pH 8.6 with 1:50000 merthiolate was used for the reacting medium. The reagents to be subjected to electrophoresis were mixed with equal volumes of 2% agar in 0.04 ionic strength barbital buffer at 45°C before placing in the appropriate well. The electrophoresis was carried out at 150 volts for 45 minutes at 4°C yielding an average milliamperage of 4 ma/cm during the run. Readings of precipitation were made 5, 24, 48 and 72 hours after filling channels with appropriate reagents at room temperature.

Results. Rabbits immunized with the glob-

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† The term "submaxillary" has been retained in preference to the now accepted term submandibular because of the usage in our original report.

‡ Special Difco "Noble Agar."

TABLE I. Gel Precipitation Test: Reactions of Rabbit Submaxillary Antisera with Various Rabbit Organ Extracts and Whole Rabbit Sera.

Rabbit organ extracts*	Rabbit submaxillary antisera				
	1565	2002	2014	2018	2026
Submaxillary	+†	+	+	+	+
Parotid	+	+	—	+	—
Sublingual	+	—	—	—	—
Other organs*	—	—	—	—	—
Normal rabbit sera a-c and e-r	—	—	—	—	—
Normal rabbit serum d	—	—	—	+‡	—

* Protein concentrations are: submaxillary–0.125, parotid–2.5, and sublingual–1.7. Other organs tested and these concentrations are: brain–2.1, liver–8.3, kidney–4.9, ovary–4.6, adrenal–5.2, spleen–6.4, pancreas–6.7, thyroid–1.4, tongue–3.6, in grams %. All organ extracts and sera were tested undiluted and at 1/3 and 1/9 dilutions.

† Method of reading:
+ = line of precipitation
— = no visible precipitation

‡ Antiserum 2018 formed a band of precipitation with normal rabbit serum "d". This is interpreted as a precipitin reaction with a serum isoantigen. It appears to be due to an antibody which is distinct from the one reactive with rabbit submaxillary extract.

ulin fraction of rabbit submaxillary extracts develop complement fixing antibodies to the rabbit submaxillary gland comparable to those obtained previously in response to immunization with rabbit submaxillary suspensions(1). Some antisera yielded precipitation reactions with the immunizing antigen(s). The question arose whether the observed precipitation reaction occurred only with submaxillary antigens or with extracts of other tissues or with serum proteins. Ouchterlony tests were, therefore, performed with antigens prepared from rabbit submaxillary, parotid and sublingual glands as well as 9 other rabbit organs and normal sera of 18 individual rabbits. All antigens were tested undiluted at 1:3 and 1:10 dilutions. The protein concentrations of the undiluted antigens of other rabbit organs were at least 10-fold greater than that of the submaxillary extract. The results obtained are summarized in Table I.

As shown in Table I, one of the 5 positive sera, 1565, yielded a precipitation reaction with all 3 of the salivary glands tested, 2 of the sera (2002 and 2018) reacted with extracts of the submaxillary and parotid glands, but not with sublingual antigens under the test conditions; and 2 of the sera (2014 and 2026) reacted only with the submaxillary antigen. These positive antisera yielded precipitin reactions with none of the other organs tested. One of the 18 normal rabbit sera yielded a single line of precipitation with one of the rabbit antisera (2018). The precipitin line against this serum yielded a reaction of non-identity on Ouchterlony plates with the precipitin reaction opposite the submaxillary antigen. Thus, the reaction of serum 2018 with normal rabbit serum which appeared to be that of a serum isoantibody is distinct from the submaxillary antibodies. The results summarized in Table I, therefore, point to the organ specificity of the precipitin reactions obtained with rabbit antisera to rabbit submaxillary antigens for salivary gland antigens.

Most rabbits (11 of 16) immunized with 2.5 mg of submaxillary globulin gave a weak though definite skin reaction when tested with submaxillary globulins. Tests were performed by intradermal injections of 1 μg doses of globulin. The positive reactions consisted of areas of erythema which appeared in 18 to 24 hours and usually subsided or disappeared in 48 hours. Control injections with liver globulins did not yield such responses. However, histologic studies of submaxillary glands of these rabbits revealed only minimal pathologic changes (focal infiltrations of round cells) which appeared to be of doubtful significance.

The question arose whether the observed salivary gland specific precipitation reaction is due to the autoantibodies observed previously by complement fixation and hemagglutination reactions. Four of the precipitating rabbit antisera to rabbit submaxillary antigens were selected for testing with extracts prepared from the individual submaxillary glands of the antibody producing rabbits. The sera were concentrated to half their original volume. Protein concentration of the submaxillary extracts was adjusted to 4%. Each serum was tested against serial 4-fold dilutions of the extracts (4.0, 1.0, 0.25, and 0.06% protein respectively). The submaxillary glands of 2 normal rabbits were tested

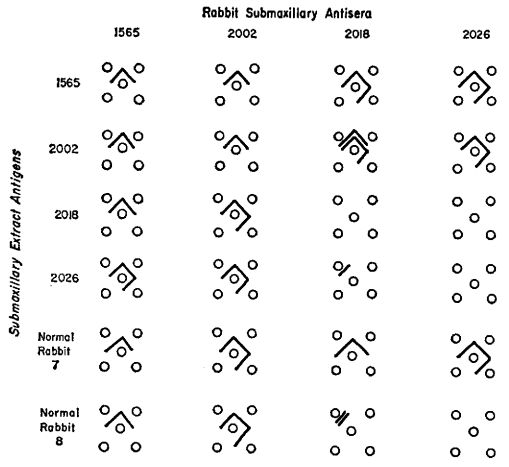

FIG. 1. Gel precipitation test: Reactions of rabbit submaxillary antisera with extracts of individual rabbit submaxillary glands. Schematic representation of the test. Peripheral circles represent wells containing serial dilutions of individual submaxillary extracts and central circle represents the well containing 2-fold concentrated antiserum. Upper left well contains 4 g % protein, upper right well contains 1 g % protein, lower right well contains 0.25 g % protein and lower left well contains 0.06 g % protein in each test. The lines between wells represent bands of immune precipitate.

in addition to those of the antibody producing rabbits. The precipitin lines observed in the test of rabbit antisera with extracts of their own and other individual submaxillary glands are shown as line drawings in Fig. 1. Rabbit antisera 1565 and 2002 formed precipitin bands with all of the extracts against which they were tested including those of their own submaxillaries. Thus 2 sera appear to contain precipitating *auto*antibodies. On the other hand, the other 2 sera, 2018 and 2026, yielded precipitin reactions with only some of the extracts. They failed to react with extracts of their own submaxillaries. Therefore, these 2 sera contain precipitating *iso*antibodies. It is noteworthy that at least 2 distinct isoantibodies appear to be involved. The appearance of these reactions is illustrated in Figs. 2, 3, and 4. Fig. 2 is a photograph of the gel diffusion precipitation reactions obtained with the autoantibodies of serum 1565. A single line of precipitation developed with each of the submaxillary extracts tested including the one of the antibody producing rabbit (well #6). Fig. 3 on the other hand, illustrates the isoantibody reactions of serum 2018 with the same 6 antigens. This serum yielded lines of precipitation with extracts of the submaxillaries of rabbit 1565 (well #6), rabbit 2002 (well #1), and normal rabbit 7 (well #2) but none with the extract of normal rabbit 8 (well #3), rabbit 2026 (well #4), and rabbit 2018, the antibody producer (well #5). The antigens involved in the reactions with the auto- and isoantibodies were further distinguished by simultaneously testing the two types of antibodies with a globulin prepared from pooled rabbit submaxillary glands. Fig. 4 illustrates the reactions obtained in such a test. The precipitin lines obtained with serum 1565 and 2026 appeared to give reactions of *non*-identity. Complement fixation tests with antigen dilutions yielded comparable autoantibody reactions with serum 1565 and isoantibody reactions with serum 2026. Only autoantibodies occurred in the original sera(1).

The antigens which react with the auto- and isoantibodies were further characterized by subjecting pooled rabbit submaxillary globulin to immunoelectrophoresis. Sera containing precipitating auto- and isoantibodies were placed in alternate troughs. Fig. 5 illustrates results obtained in such an experiment. After the electrophoresis of the antigen (wells a and b) the precipitating antisera of rabbits 2018, 1565, and 2026 were added to troughs 1, 2, and 3 respectively. It can be seen that the crest of the bands formed by autoantibody containing serum 1565, and those formed by isoantibody containing sera 2026 and 2018 are at different positions. This again indicates that the auto- and isoantibodies are directed to different antigens. The double band formed by antiserum 2018 suggests that antibodies against at least 2 submaxillary isoantigens may be present in this serum. Immunoelectrophoretic analysis of the above iso- and autoantisera demonstrated that the antibodies in the rabbit serum have an electrophoretic mobility similar to that of rabbit gamma globulin.

Rabbit saliva also yielded precipitin lines in gel diffusion precipitation tests with both types of antisera. To determine whether these precipitin lines were due to the same antigen found in the salivary glands, submaxillary extracts and saliva were tested simultaneously with the precipitating anti-

bodies. Fig. 6 illustrates the type of reaction obtained in such an experiment. The line of precipitation obtained with antiserum 1565 (well #3) opposite saliva (well #1) merges with the line opposite the submaxillary extract (well #2). This reaction of identity indicates that the organ specific antigen found in the submaxillary gland is secreted in the saliva. Further tests of this type also revealed that the serum of rabbit 2002 yielded precipitin lines with the animal's own saliva.

The species specificity of the rabbit submaxillary antibodies was studied with precipitation and complement fixation methods. Three sera known to contain autoantibodies, 1565, 2002, and 2014, as well as 2 isoanti-

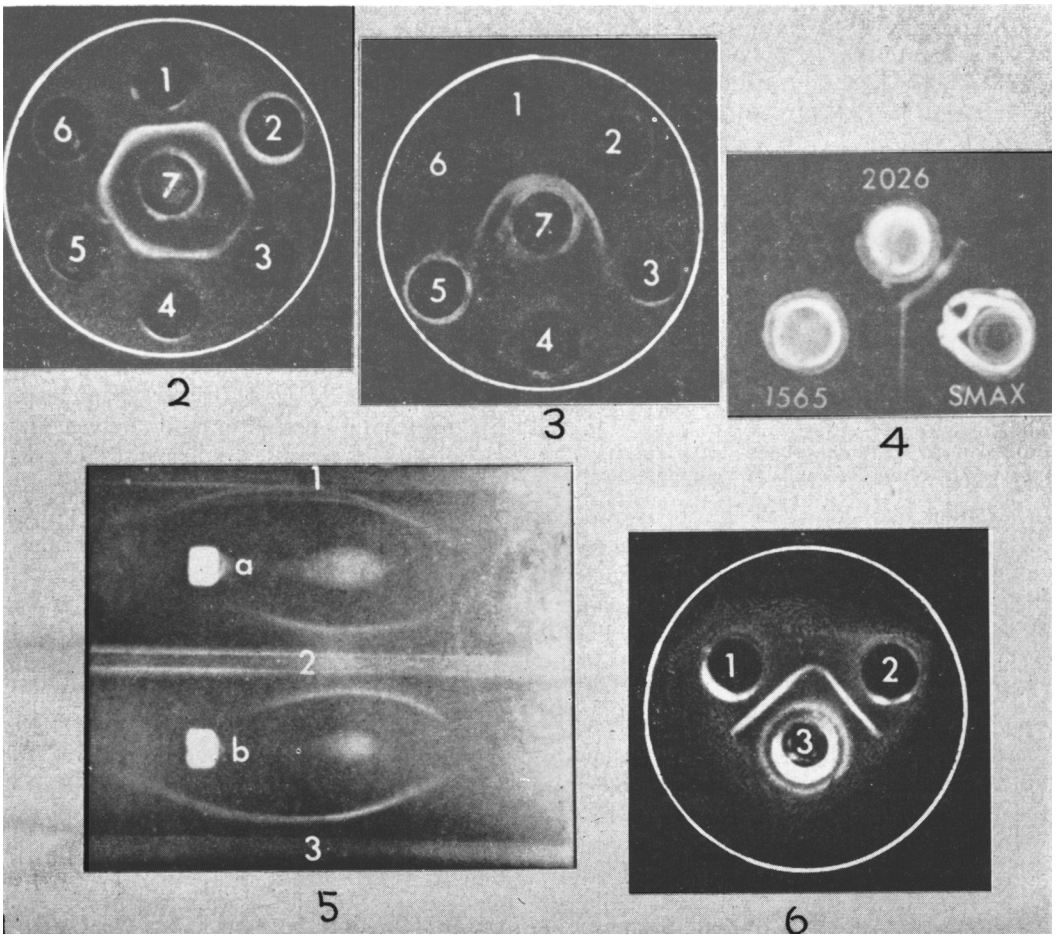

FIG. 2. Well #1, rabbit 2002 submaxillary extract; well #2, normal rabbit #7 submaxillary extract; well #3, normal rabbit #8 submaxillary extract; well #4, rabbit 2026 submaxillary extract; well #5, rabbit 2018 submaxillary extract; well #6, rabbit 1565 submaxillary extract; well #7, rabbit submaxillary antiserum 1565.

FIG. 3. Wells #1-6 contain the same antigens as wells #1-6 in Fig. 2. The center well (#7) contains rabbit submaxillary antiserum 2018. Note difference in reaction patterns here from those of autoantibodies in Fig. 2.

FIG. 4. Serum 2026 in upper well contains precipitating isoantibodies while serum 1565 in lower left well contains autoantibodies to submaxillary globulin (lower right well).

FIG. 5. Immunoelectrophoresis: Rabbit submaxillary globulin (5 g %) in wells a and b were electrophoresed, antisera were added; trough 1, antiserum 2018, trough 2, antiserum 1565, and trough 3, antiserum 2026. Note difference in mobility of antigens reactive with auto (2) and iso (1 and 3) antigens.

FIG. 6. Reaction of rabbit submaxillary antiserum with rabbit saliva and rabbit submaxillary gland extract: well #1—pooled, concentrated rabbit whole saliva, well #2—pooled rabbit submaxillary extract and well #3—rabbit submaxillary antiserum 1565.

body containing sera, 2018 and 2026, were tested with pooled submaxillary extracts of rabbit, human, monkey, dog and guinea pig origin by the 2 test methods. Initial studies revealed precipitation reactions (but not complement fixation) with human monkey salivary glands but no reactions with other mammalian salivary glands or with other tissues of monkey or human origin in sera: 2002, 2014, 2018. This suggested the presence of organ specific heteroantibodies. However, further studies indicated that these precipitating antibodies were directed against an isoantigen of primates and that this antigen was present not only in the salivary glands but also in the serum of some but not of other individuals. This antigen was found to be identical with the rabbit serum antigen to which antibodies had been demonstrated previously (Table I), though at that time only 1 of the 3 sera reactive with human sera yielded precipitin reactions with the rabbit sera tested. Apart from these antibodies to serum proteins, the auto- and isoantibodies to rabbit salivary gland antigens appear to be not only organ but also species specific within the limits of the species tested.

Discussion. Pathologic changes in salivary glands of immunized rabbits comparable to those observed in Sjögrens syndrome could not be demonstrated in our studies. According to a preliminary report by Waterhouse (5), focal adenitis was produced in salivary and lacrimal glands of guinea pigs by an immunization procedure comparable to the one used in our study. However, as the author states, the number of animals (3 adjuvant controls and 7 positives) was too small to reach any firm conclusions. It appears that while serologic responses to, but no conclusive evidence of pathologic changes in, salivary glands have thus far been observed in experimental animals that the converse is true of humans. The pathologic changes of salivary glands in Sjögrens syndrome have been likened to those of autoimmune thyroiditis(6,7); but efforts to demonstrate salivary gland specific antibodies in humans have thus far been unsuccessful(8). It may be that only cellular immune responses are operative in the pathogenesis of the disease and that humoral antibodies such as those observed in our studies are not "pathogenic." If and when significant, specific, pathologic changes of salivary glands are elicited in experimental animals by immunologic means, it will be of considerable fundamental importance to determine whether they are accompanied by such immune responses as have been demonstrated in our studies of rabbits.

The demonstration of precipitating autoantibodies to submaxillary antigens in rabbit antisera to rabbit submaxillaries was anticipated on the basis of previous studies of such antisera by complement fixation and tanned cell hemagglutination tests(1). Isoantibodies specific for pancreatic antigens have been found in studies of immune responses of rabbits to injections of pooled rabbit pancreas antigens, as demonstrated by complement fixation and gel precipitation tests(9). However, unlike the submaxillary system no autoantibodies (to pancreas) could be detected.

Summary. Some rabbits immunized with 2 M ammonium sulfate precipitable fraction of rabbit submaxillary gland extract in Freund adjuvant developed complement fixing and precipitating antibodies and a cutaneous hypersensitivity of the delayed type, specific for immunizing antigen. Sera which yielded salivary gland specific precipitation and complement fixation reactions also yielded comparable reactions with rabbit saliva. Studies of the complement fixation and precipitation reactions pointed to the presence of distinct auto- and isoantibodies in different immune sera. The relevant observations may be summarized as follows. 1. *Autoantibodies.* Some rabbit antisera fixed complement and yielded lines of precipitation in Ouchterlony plates with extracts and globulins of submaxillaries of all rabbits tested including the antibody producing rabbits' own submaxillaries and saliva. 2. *Isoantibodies.* a) Some rabbit antisera yielded precipitation reactions with pooled rabbit submaxillary globulins and with antigens prepared from some individual glands but not with the submaxillary extracts of the antibody producing rabbits. b) Some isoantibody containing sera also yielded complement fixation reactions with some rabbit submaxillary extracts but not with antigens from the antibody producing rabbits. c) Immunoelectrophoretic stud-

ies revealed that the antigen(s) reactive with autoantibodies and those reactive with isoantibodies have distinct electrophoretic mobilities. d) Gel diffusion precipitation tests of auto- and isoantibody containing sera appeared to yield reactions of non-identity.

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Parotid Saliva-Serum Ratios of Ca^{47} in Man Following Intravenous Administration of the Isotope.* (30001)

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The present study was designed to investigate the salivary transport of calcium in the human by determining the specific activity ratios of Ca^{47} in the blood and parotid saliva following intravenous injection of the isotope. Only Greenberg *et al*(1) appear to have reported on the blood to saliva specific activity ratios of Ca^{47} in the human. However, no details were given.

Methods. Five normal males, 21-39 years of age and 5 normal females, 24-36 years old received 10-15 μc Ca^{47} intravenously before breakfast. At frequent intervals during the next 24 hours, as shown in Table I, blood was taken from the contralateral antecubital vein, and parotid saliva was collected according to previously described technics(2-4). Calcium was determined by flame photometry according to MacIntyre(5) and expressed in grams per 2.0 ml. Ca^{47} was determined on 2 ml of serum and parotid saliva in a well scintillation counter. Discriminator settings were selected to give optimal statistical accuracy with a counting error no greater than 1%. Energies below 400 kev were eliminated by a single channel gamma ray spectrometer to exclude the gamma emission of Sc^{47}. In each instance, aliquots of the administered Ca^{47} test dose were also counted using identical geometric conditions. Specific activity in saliva and serum is defined as the fraction of injected dose of Ca^{47} per gram of calcium. These are plotted for both males and females in Fig. 1.

Results. For both males and females, the salivary calcium varied from 2.5 mg% to 3.3 mg%, and blood calcium from 9.2 mg% to 10.0 mg%. The ratio of saliva to serum specific activities of Ca^{47} of the males approached unity one hour after intravenous administration of the isotope and was maintained for 24 hours.

The mean salivary-serum specific activity ratios in the females, in general, approached unity during the 24-hour study. A high salivary-serum specific activity ratio of 1.78 was obtained in one patient (Table I) 30 minutes following Ca^{47} infusion. The females appeared in fact to "concentrate" Ca^{47} in the

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