A 'New' Saliva Substance, Probably Inherited, and Serologically Independent of ABH, Lewis, and Sd^a Blood Group Substances

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Summary. The presence of a 'new' inherited substance has been detected in Caucasian, Bantu, and Chinese saliva samples, meconium, and gastric juice using the haemagglutinins of *Clostridium botulinum*, types C and D. It is serologically independent of ABH, Lewis, and Sd^a secretor status; at present it does not appear to be related to blood group systems and it is absent from the saliva of 7.14% of the Caucasian population. The structure of the substance may be related in some way to human gangliosides.

The presence of water soluble ABH and Lewis 'blood group active substances' in tissue fluids and secretions has been known for some time (for a review see Race and Sanger, 1968). More recent is the discovery of the antigen Sd^a in the saliva of Sd(a+) people (Macvie, Morton, and Pickles, 1967; Renton *et al*, 1967). These substances were so named because the corresponding antigens were first detected on erythrocytes.

Blood group specific lectins and protectins (for a recent review see Sharon and Lis, 1972) have proved of considerable value in the determination of the immunodominant sugars responsible for blood group activity, since they are specifically inhibited by monosaccharides, whereas human or animal antibodies are complementary to larger determinants.

Gold and Phelps (1972) have broadened the concept of lectins and protectins to include non-antibody haemagglutinins present in a wide range of organisms: viruses, bacteria, fungi, plants, sponges, snails and fishes, and have suggested the descriptive term 'receptor specific proteins' (RSP) for these substances.

During work with bacterial haemagglutinins from *Clostridium botulinum* types A, C, and D Balding *et al* (1973) found that while many saliva samples inhibit type C or type D in haemagglutination-inhibition tests, a few did not. This finding prompted us to determine whether the active in-

hibiting substance was related to ABH, Lewis, or Sd^a secretor status, and if not, whether it was inherited. The results of this investigation are reported here.

Materials and Methods

The *Clostridium botulinum* haemagglutinins used here have been described by Balding *et al* (1973).

Anti-H lectin was prepared from *Ulex europaeus* seeds as described by Dunsford and Bowley (1967).

Human red cells and saliva, except as detailed otherwise below, were obtained from the Regional Transfusion Centre, Bristol. Red cells with the following rare phenotypes were a gift from Dr R. R. Race, The Lister Institute, London: Co(a-), p^k, K₀, Js(b-), Kp(a+b-), U-. ABH and Lewis (*lelesese*) non-secretor saliva was a gift from Dr D. Voak, Regional Blood Transfusion Centre, Cambridge. Bantu salivas were a gift from Dr P. Brain, Natal Institute for Immunological Research, Durban. Salivas from Bristol families were collected by arrangement with Professor R. C. Wolfenden, Chief Public Health Officer for Bristol. Neonatal salivas and meconium from newborns less than 24 hours old were obtained from Southmead Hospital Maternity Unit, Bristol. Salivas from known monozygotic twins, and the Chinese saliva samples were obtained by the authors from suitable donors. Normal gastric juice was provided by Dr T. S. Low-Beer, Department of Medicine, University of Bristol. Three specimens of blood group specific glycoproteins with varying quantities of sialic acid were a gift from Professor W. M. Watkins, The Lister Institute, London.

Isotonic phosphate buffered saline pH 7.0 (PBS) was used for all cell suspensions and for dilutions.

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Cell samples, collected in ACD, were washed three times in PBS and used as 3% suspension in PBS as soon after collection as possible but never after more than one week.

Saliva samples were boiled in a water bath for 15 min, centrifuged hard, and the clear supernatant was used in serological tests. Gastric juice, neutralized by the addition of 0.1 M NaOH was treated in the same way as the saliva samples, except that an aliquot was also used un-boiled. Meconium was diluted 1 in 4 in PBS, vigorously shaken for several minutes and centrifuged to remove debris. The supernatant was separated and centrifuged until no longer opaque and then used in serological tests.

Serological Tests. Haemagglutination (H) and haemagglutination-inhibition (H/I) tests were performed as described by Dunsford and Bowley (1967). Master dilutions of the substance to be tested were made and 0.05 ml of each dilution was mixed with 0.05 ml of haemagglutinin (4 haemagglutinating units, checked with each series of tests) in 5 × 40 mm tubes; after 30 min incubation at 20° C, 0.05 ml of the red cell suspension was added. Tests were read microscopically after 2 hours incubation at 20° C. Inhibition titre was recorded as the greatest dilution of inhibitor to give complete inhibition of agglutination. Agar-gel double diffusion tests were performed according to the method of Ouchterlony (1958).

TABLE I
THE INHIBITION OF *CLOSTRIDIUM BOTULINUM*
TYPE C AND TYPE D BY SALIVA

Saliva	ABO Blood Group	Inhibition Titre*	
		Type C	Type D
ABH secretor	A ₁	1024	32
	A ₂	1024	nt
	B	512	32
	A ₁ B	64	nt
	O (i)	1024	32
	O (ii)	32	16
ABH non-secretor	A ₁	64	nt
	B (i)	256	nt
	B (ii)	0	0
	A ₁ B	1024	nt
	O	64	4
ABH and Lewis non-secretor	O	32	8
Sd ^a strong secretor	—	512	8
Sd ^a medium secretor	—	2048	128
Sd ^a non-secretor	—	1024	256
Chinese	—	128	8
Bantu (i)	—	1024	32
Bantu (ii)	—	0	0
Neonatal	—	128	16
Monozygotic twin (i)	—	1024	16
Monozygotic twin (ii)	—	1024	16

* The number indicates the reciprocal of the greatest dilution of saliva to give complete inhibition of a minimal agglutinating dose of agglutinin.

nt = not tested.

Results

In total H/I tests with 100 Caucasian, 30 Bantu, and one Chinese saliva samples, two gastric juice, and three meconium samples have been performed. Results for salivas are shown in Table I. Representative titres only are given for salivas of different blood group, race, etc. Tests with type D were limited by the short supply of this valuable purified material.

Salivas of A, B, H, Lewis, and Sd^a secretors and non-secretors inhibit both type C and type D, showing that the inhibiting substance is independent of A, B, H, Lewis, and Sd^a secretor status. Strong inhibition by the saliva of one group B non-secretor, the lack of inhibition by another and the inhibition of both type C and type D by the *lelesese* saliva lacking ABH and Lewis substances support this conclusion.

The H secretor status of ABH and Lewis secretor and non-secretor salivas was also determined with anti-H lectin from *Ulex europaeus*, in case the different inhibition titres with type C or type D should reflect different concentrations of all soluble substances in saliva, due to varying rate of secretion, individual differences in secretor level, time of day etc. Sturgeon, McQuiston, and van Camp (1973a and b) have described the influence of such physical variations on secretor level, and Caldwell and Pigman (1965) have also reported quantitative and qualitative differences in human saliva glycoproteins. Results are not in Table I but each saliva showed normal H secretor status for its group, indicating that the possible variation was insignificant in our tests. The saliva samples of family B (see below) were tested in a similar way, also showing normal H secretor status.

It is possible that there may be not one but two new substances detected by type C and type D, respectively, as suggested by the saliva inhibition titres; we have not, however, been able to confirm identity, partial identity, nor non-identity of their receptors in agar gel diffusion tests, as neither type C nor type D appear to form a precipitate with a soluble receptor substance.

The inhibiting substance is present in Caucasian, Bantu, and Chinese salivas. In Fig. 1, the graphs show the number of salivas giving a particular inhibition titre with type C against that inhibition titre. Fig. 1 shows the results with unrelated Caucasians and unrelated Bantus; the level of secretion appears to be less in the latter group. The bimodal distribution of titres is probably due to differences in zygosity; the peak for higher inhibition titres corresponds to homozygotes (Caucasians 1 in 1024, Bantus 1 in 100); the peak for

FIG. 1. Inhibition titres with *Clostridium botulinum* type C, and unrelated saliva samples. (No inhibition by three Caucasian samples or one Bantu sample.)

lower inhibition titres corresponds to heterozygotes (Caucasians 1 in 100, Bantus 1 in 16).

The symbols *Se* and *se* are reserved for the genes governing secretion of ABH and Lewis substances; the symbols *Sal^{Cbc}* and *Sal^{Cbd}* are therefore proposed for the genes governing the secretion of the substances, provisionally named CbC, and CbD, inhibiting type C and type D, respectively.

The provisional gene frequencies have been calculated from the results with unrelated Caucasian saliva samples as shown in Fig. 1.

Out of 42 Caucasians, three were non-secretors, 20 were heterozygotes, and 19 homozygotes. Gene frequencies are as follows:

$$Sal^{Cbc} : 0.7328$$

$$sal^{Cbc} : 0.2672.$$

There are therefore 92.86% secretors (53.7% homozygotes and 39.16% heterozygotes) and 7.14% non-secretors. Experimental results are in agreement with these values.

Results with neonatal saliva samples show that the substance is well developed at birth. Several pairs of twins were investigated: known monozygotic twins gave identical inhibition titres—pointing to the possibility of including secretion of this new substance in investigations of the zygosity of human twins. Salivas from another pair of twins in family B (see below) each gave different inhibition titres; their H secretor status was also different, indicating that they are dizygotic. Two other pairs

TABLE II
THE INHIBITION OF *CLOSTRIDIUM BOTULINUM* TYPE C BY VARIOUS SUBSTANCES

Substance	Type C Inhibition Titre*
Gastric juice (boiled)	32
Gastric juice (non-boiled)	128
Meconium	128
Blood group specific glycoproteins (2 mg/ml) containing:	
15% sialic acid	1
4% sialic acid	0
1.3% sialic acid†	1

* The number indicates reciprocal of greatest dilution of substance to give complete inhibition of a minimal agglutinating dose of agglutinin.

† Values below 2%, probably represent sialic acid free material.

of twins have also been tested; on the basis of our results one pair appears to be monozygotic, the other pair dizygotic.

Results of type C H/I tests with other substances are shown in Table II. The three meconium samples and the gastric juice samples contain the inhibiting substance. One neutralized gastric juice sample was tested to see if boiling (to inactivate enzymes) affected the inhibitor; both the boiled and unboiled samples inhibited haemagglutination.

It has been found (Balding *et al.*, 1973) that the structure or configuration of the receptor for type C and type D probably depends on the presence of N-acetyl-neuraminic acid (NANA); three specimens of ABH blood group specific glycoproteins with varying quantities of sialic acid were therefore tested. None significantly inhibited type C, again showing that the substance inhibiting type C is independent of the ABH active glycoproteins irrespective of their sialic acid content. H/I tests with salivas of 10 Caucasian families were performed with type C (Table III). One of these families, family B, proved very interesting and was also investigated with type D and with anti-H lectin; results are shown in Table IV. In this family the father is heterozygous for the gene controlling secretion of the inhibiting substance. The saliva sample from the mother gave complete lack of inhibition. Results with the saliva of the children strongly suggest that the inhibiting substance is a dominant inherited character. Pedigrees of family B with type C and type D are shown in Figs. 2a and 2b.

No significant difference in agglutination titres were found in tests with 100 native (non-enzyme treated) random human red cell samples, or with a panel of red cells showing presence or absence of the following common antigens: A, B, O, C, D, E, c, e, M, N, S, P₁, P₂, K, Le^a, Le^b, Fy^a, Fy^b, Jk^a, Jk^b. Red cells with the following rare phenotypes,

TABLE III
RESULTS OF INHIBITION TESTS WITH *CLOSTRIDIUM BOTULINUM*
TYPE C AND SALIVA SAMPLES FROM CAUCASIAN FAMILIES

Family	Inhibition Titre*									Others
	Father	Mother	Children							
			1	2	3	4	5	6	7	
A	128	128	64	16	16	32	8			
B	128	0	16	0	0	16	Twin 0	Twin 64	32	
C	1024	64	1024	128	64	128				
D	1024	64	1024	1024	128	128	256	256		
E	512	128	128	512	64	512	512			
F	32	512	256	256	128	128	32	128		Grandmother 32
G	512	128	512	512	16	512				
H	512	64	4	256	256	8	128			
I	1024	2048	2048	2048	1024	2048	512			
J	16	128	32	16	4	16	128			

* The number indicates reciprocal of greatest dilution of saliva to give complete inhibition of a minimal agglutinating dose of agglutinin.

Co(a-), p^k, Ko, Js(b-), Kp(a+b-), and U- gave, with type C and type D, titres similar to those obtained with randomly chosen red cells. Many more cell samples, lacking very common antigens must be tested before any relationship between the receptor for type C and type D and a known blood group antigen can be ruled out.

TABLE IV
RESULTS OF INHIBITION TESTS WITH
CLOSTRIDIUM BOTULINUM TYPE C AND TYPE D,
AND SALIVA SAMPLES OF FAMILY B

Family Member	ABO Blood Group	Inhibition Titres*	
		Type C	Type D
Father	A	128	32
Mother	O	0	0
Children			
1	A	16	32
2	O	0	0
3	A	0	2
4	O	16	2
5	O	0	0
6	O	64	8
7	nt	32	8

* The number indicates reciprocal of greatest dilution of saliva to give complete inhibition of a minimal agglutinating dose of agglutinin.
nt = not tested.

FIG. 2a and b. Pedigrees of family B.

Discussion

The discovery of an inherited blood group active substance secreted in saliva has previously followed discovery of the associated red cell blood group, primarily because the corresponding antibody is first detected and investigated; saliva samples are then used in H/I tests with the antibody leading in a few cases to the recognition of a secreted soluble antigenic substance. The results of such studies have proved valid even though most of the precautions described by Sturgeon *et al* (1973a and b) to minimize variables governing secretion of saliva glycoproteins have not been taken. The same is true of routine tests for detection of ABH secretors. The large titre differences observed in our tests and in family studies therefore reflect real differences in salivary secretor status which, as shown by the results with family B, are inherited as dominant characteristics. It is however conceivable, but is thought unlikely, that the titre differences are not due to zygosity but to quantitative alleles (Sturgeon and Arcilla, 1970).

In this report we describe a 'new' substance secreted in saliva recognized by using, not an antibody, but a RSP. Should no human red cell be found which fails to be agglutinated by type C or type D, the concept of an inherited 'saliva group' not related to a red cell group must be accepted.

Inhibition studies using purified human gangliosides (our unpublished observations) indicate that preferentially ganglioside GI (Klenk and Gielen, 1963; Kuhn and Weigandt, 1963a) may be the receptor for type C, and ganglioside GIV (Kuhn and Weigandt, 1963b) may be the receptor for type D on human red cells.

It has been shown that ABH antigens are glycoproteins in secretions, but may be glycolipids* on red cells. In the same way, the substance in saliva inhibiting type C and type D may be glycoprotein with a sugar chain similar to the inhibiting ganglioside, which may be the glycolipid receptor on red cells. The exact structure of the inhibiting saliva substances must await results of purification from saliva.

The presence of blood group active substances in secretions, especially saliva, has proved both genetically and biochemically important. The

substances inhibiting H by type C and type D are also found in secretions. Like the ABH substances, they may prove of interest in the study of cancer of the gastrointestinal tract, and in population studies of various races.

The finding of the inherited saliva substance reported here provokes the idea that other non-blood group specific RSP may also react with as yet undefined inherited substances in secretions.

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* ABH antigens on red cells may also be glycoproteins.