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Specific Differences in the Inhibition Titers of the Anti-H Lectins from Cytisus sessilifolius and Ulex europaeus *

CHRIS C. PLATO and HENRY GERSHOWITZ

University of Michigan Medical School, Department of Human Genetics
Ann Arbor, Michigan

In 1948 *Renkonen* [9], and a year later, *Boyd and Reguera* [3] recognized that several plant seed extracts (lectins) act as haemagglutinins. Since that time the haemagglutinating action of the lectins has been verified by many investigators (see *Bird* [2]). It has also been demonstrated that some lectins are quite specific in their reactions with certain blood group substances (*Bird* [1], *Boyd and Shapleigh* [5], and *Cazal and Lalaurie* [6]).

The anti-H lectins are of special interest among these plant extracts, because of their usefulness in the determination of the secretor status where soluble blood group substances are present in the saliva (*Boyd and Shapleigh* [4]).

It is generally believed (see *Glynn and Holborow* [7]) that all secretors contain in their salivas detectable amounts of H substance. Some exceptions to this generality were reported by *McNeil et al.* [8] who, by using *Lotus tetragonolobus* as an anti-H source, encountered some secretors whose saliva inhibited either the anti-A or the anti-B but not the anti-H. These cases they labelled as "aberrant secretors".

In addition to *Lotus tetragonolobus*, seed from *Laburnum alpinum*, *Cytisus sessilifolius*, *Ulex europaeus*, and *Tetragonolobus purpureus* have been commonly used as sources of anti-H (see *Cazal and Lalaurie* [6]). The objectives of this investigation were: (1) to determine whether any significant differences exist between the inhibition titers of the anti-H reagents obtained from seeds of two of these plants, when tested against the same saliva; (2) to establish whether salivas from secretors of different blood groups show differences in the inhibition titers in respect to these two lectins. To this end, the inhibition titers of the salivas of persons of A_1, A_2, O, B and AB blood groups were tested against constant dilutions of *Cytisus sessilifolius* and *Ulex europaeus* extracts.

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Materials, Data and Methods

Material: The seeds used in this experiment were kindly supplied by the Botanical Gardens of the University of Lisbon, Portugal.

Reagents: Both the *Cytisus sessilifolius* and the *Ulex europaeus* anti-H reagents (which will be abbreviated as anti-H_c and anti-H_u respectively) were prepared as follows: The seeds were ground to a fine powder and the meal was mixed with saline in a ratio of ¼ by weight, (1 gm. of meal to 3 cc. of saline). The mixtures were agitated in a Kahn shaker for an hour. After settling overnight the mixtures were centrifuged for twenty minutes at 6,000 rpm. The supernatant fluid was then frozen and constituted the concentrated stock. These initial preparations of both extracts were used throughout the experiment. This was done to avoid changes in the potencies of the extracts which are introduced by the use of different seed lots. The usual deviations observed in the preparation of several stocks were also avoided by the use of only one preparation. The agglutination titers (after centrifugation) of these stock fluids tested with the same control type O cells are shown in Table I.

TABLE I

Agglutination Titers of the Anti-H Reagents Extracted from Cytisus sessilifolius *(Anti-H_c) and* Ulex europaeus *(Anti-H_u) Tested with the Same O Red Cells.*

Extract Dilutions	Agglutination Titers ½	¼	1/8	1/16	1/32	1/64	1/128
Anti-H_c	4	4	4	4	3	3	–
Anti-H_u	4	4	3	3	2	1	–

In the agglutination titers shown in Table I, as well as in the saliva inhibition titers, it has been observed that the *Cytisus* extract exhibits a threshold effect. That is, upon progressive dilutions, the anti-H_c loses potency abruptly instead of gradually, as does the *Ulex* extract. In the inhibition tests, this holds true regardless of whether the salivas have been titrated against constant dilutions of anti-H_c, or a constant saliva concentration has been tested against varying dilutions of the extract.

For testing, the anti-H_c and anti-H_u stocks were diluted to 1/50 and 1/20 respectively. At these dilutions, both the reagents had been inhibited by 1/512 dilution of the control O saliva.

Salivas: Saliva specimens were collected at random from fifty individuals of each of five ABO blood groups (A_1, A_2, O, B, and AB) with a total number of 250 specimens titrated and tested. The AB group consisted of 42 A_1B and 8 A_2B.

Methods:

(1) Two-fold dilutions of each saliva were prepared in duplicate in .85% saline solution. The dilutions in each set ranged from ½ to 1/1024 (ten tubes with one drop of diluted saliva in each).

(2) One drop of $^1/_{50}$ dilution of anti-H_c was added to each tube of the first set and one drop of anti-H_u at $^1/_{20}$ to each of the tubes of the second set. They were then left to incubate for twenty minutes at room temperature.

All fluids were delivered to the tubes using a single pipette, made by imbedding a steel, flat tip, 18 gauge needle in a glass tube. In this manner, although drop size was not measured, it could be assumed that there would be very little variation in volume between tubes.

(3) One drop of 2% suspension of "control" O red cells were added to each of the twenty tubes and incubated for twenty additional minutes.

(4) After centrifugation for fifteen seconds at 2,000 rpm. the tubes were read, and the inhibition titers were recorded. For the anti-H_c, the dilution which gave a reaction of 2 or more was taken as the end point. Any "+" agglutination was taken as the end point for the anti-H_u. These different end points were chosen because of the threshold demonstrated in the inhibition of the *Cytisus* extract.

From time to time during the experiment, samples from both reagents were tested against the control O saliva to determine the repeatability of the test. At no time did the scores for the control saliva vary by more than one dilution from any other score in the repeatability series.

Results and Discussion

The results of the inhibition tests are summarized in Table II and Figure 1. For convenience in the computations of the means and the standard deviations, the saliva dilutions were coded with scores from 1 to 11. Score "1" represents the ½ titer, 10 the $^1/_{1024}$ and score 11 was substituted for titers of over $^1/_{1024}$.

An examination of the means of the rows in Table II indicates that there is considerable difference between blood groups in the potency of the H substance found in salivas. O salivas demonstrate the highest potency of substance, with A_2, A_1, B and AB following in that order. This relationship in strength of H is noticed independently of the type of anti-H used. However, even though the trend is the same with both reagents, there is much variability between groups with respect to H_c and H_u. (These last notations represent the reactivity of a saliva to each of the specific lectins, anti-H_c and anti-H_u.) The variation in the potency of the H substance is mainly due to the extreme types, O and A_2 on one hand and B and AB on the other. The O and A_2 salivas seem to inhibit the anti-H_u better than they do the anti-H_c. The contrary is observed in the behaviour of B and AB salivas, which inhibit the anti-H_c better than they do the anti-H_u. The components of Table II have been arranged in the form of frequency histograms and are presented in Fignre 1. Start-

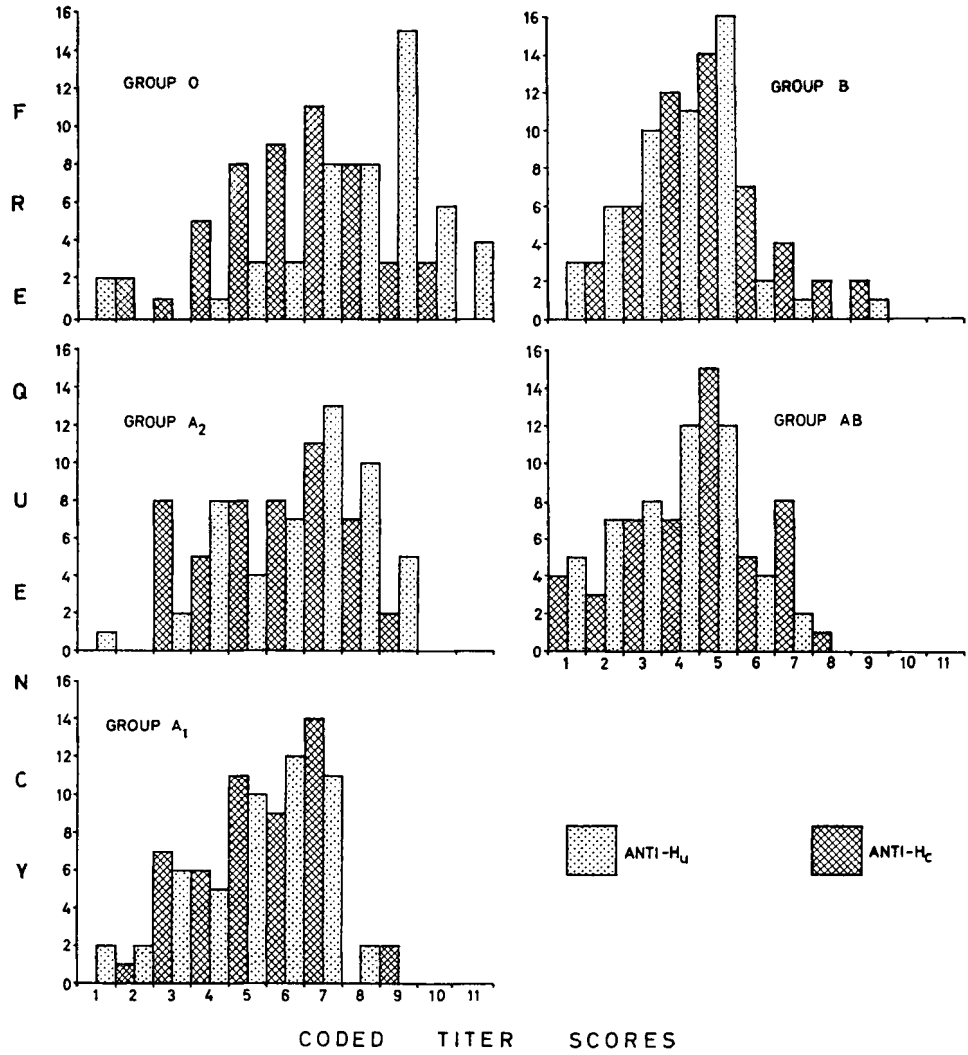

Fig. 1. Frequencies of the inhibition titer scores of salivas from persons of the several blood groups.

ing from the distributions of the O group on top, down to the AB at the bottom; note the tendency for a shift of the modes from higher H titer to lower. However, the changes in the reaction with the anti-H_u seem to be more variable than the ones with anti-H_c. Thus, the mean titer of the H_u is higher than that of H_c in the groups of high

TABLE II

Frequencies of the Inhibition Titers of Saliva of the Five Blood Groups, Tested with Both Ulex europaeus *and* Cytisus sessilifolius.

Saliva titer		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048			
Coded scores		1	2	3	4	5	6	7	8	9	10	11	N	$\bar{X}$	s
Cytisus															
$\bar{x}=5.424$	O	0	2	1	5	8	9	11	8	3	3	0	50	6.38	1.92
	A_2	0	0	8	5	8	8	11	8	2	0	0	50	5.82	1.80
	A^1	0	1	7	6	11	9	14	0	2	0	0	50	5.44	1.63
	B	0	3	6	12	14	7	4	2	2	0	0	50	4.92	1.67
	AB	4	3	7	7	15	5	8	1	0	0	0	50	4.56	1.82
Ulex															
$\bar{x}=5.440$	O	2	0	0	1	3	3	8	8	15	6	4	50	7.96	2.20
	A_2	1	0	2	8	4	7	13	10	5	0	0	50	6.34	1.92
	A^1	2	2	6	5	10	12	11	2	0	0	0	50	5.18	1.75
	B	3	6	10	11	16	2	1	0	1	0	0	50	3.94	1.56
	AB	5	7	8	12	12	4	2	0	0	0	0	50	3.78	1.60

H secretion (O and A_2) on one hand, while on the other hand it is less than the mean titer of H_c in the groups with low H secretions (B and AB). This bi-directional change in potency can hardly be attributed to a quantitative difference between the two anti-H reagents, nor can it be attributed to quantitative variation of Le^b, with which anti-H may react, among the salivas. Mere quantitative variation would not have resulted in a bi-directional change of potency. It would seem that the two anti-H reagents vary in specificity not only from group to group, but also within groups. Their differences in specificity may also be demonstrated by comparing the inhibition titer of each saliva against both of the reagents (Figures 2 and 3 and Table III).

Each saliva was titrated against constant dilutions of both anti-H_c and anti-H_u and the respective inhibition titers were recorded in scatter diagrams (Figure 2) for each blood group separately. If both reagents were equipotent for the same salivas, then the frequencies should be distributed along the 45° diagonal as is the case in the group A_1. Concentrations of points above the regression line denote

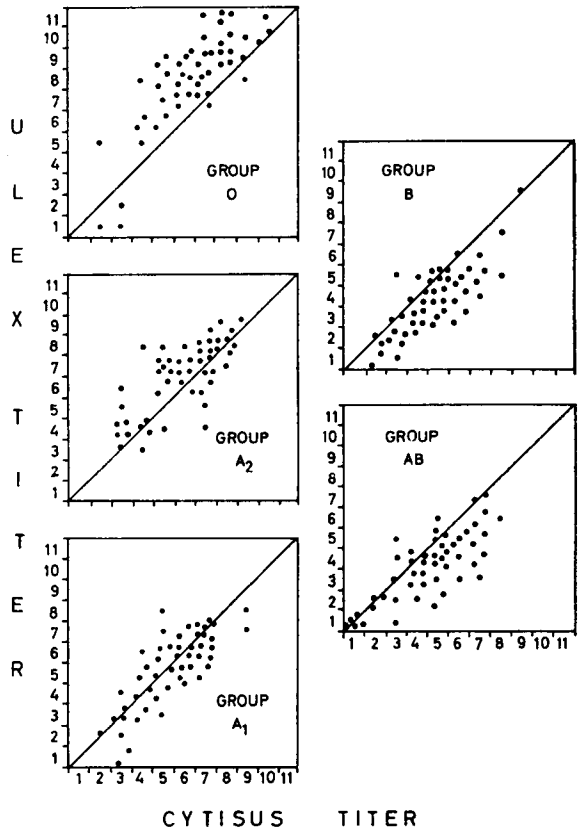

Fig. 2. Scatter diagrams of the inhibition titer scores for each blood group. Salivas were titrated against a constant dilution of *Cytisus* and *Ulex* extracts.

higher potency of H_u whereas the concentrations below the line indicate that H_c is more potent.

The comparisons presented in Table III were again based on inhibition titers of the anti-H_c and anti-H_u. In this case, however, the actual difference in titer for each saliva was studied. Thus, if for the same specimen the inhibition titers were identical, the score zero (0) was assigned. For salivas in which the titer of H_c was higher than H_u by two dilutions, the score two (2) was placed under *Cytisus* on the right side of the zero. If H_u was higher, the appropriate scores were entered at the left side of the zero. Again the scores were coded for convenience. This classification enables us to test for the significance

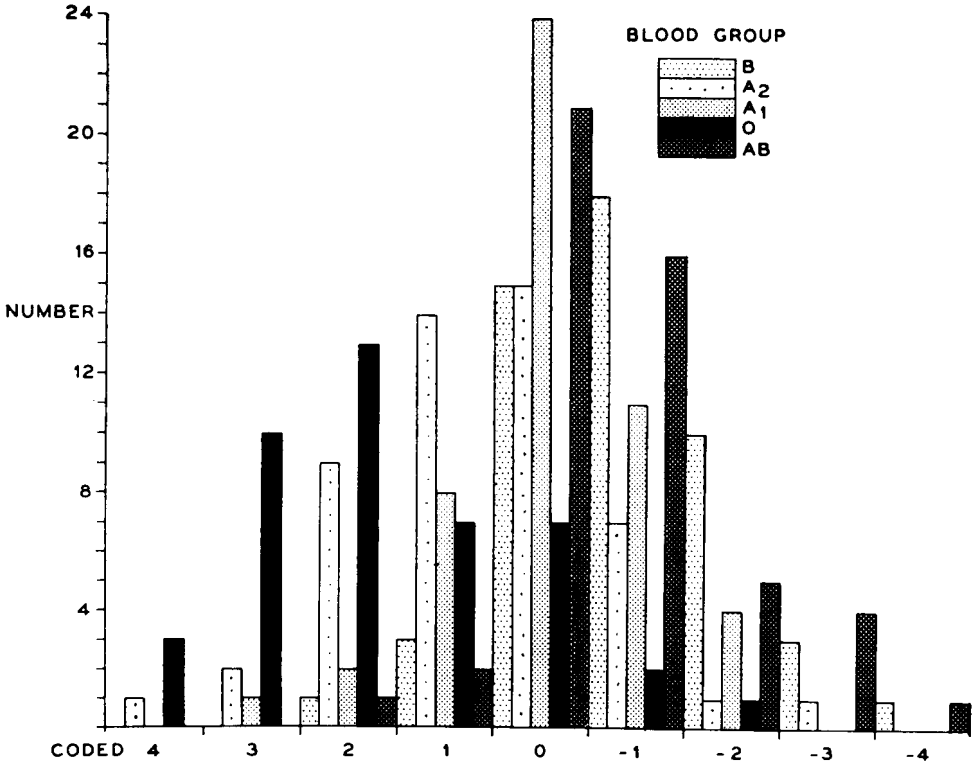

Fig. 3. Frequencies of the differences between the inhibition titers of *Cytisus* and *Ulex* extracts for all five blood groups.

TABLE III

Frequencies of the Differences between the Inhibition Titers of Anti-H_c and Anti-H_u for All Five Blood Groups

	Ulex						*Cytisus*			Mean	s_d	t
Difference (d)	4	3	2	1	0	1	2	3	4	dev.		$\left(\frac{\bar{d}-d}{s_{\bar{d}}}\right)$
Coded scores	4	3	2	1	0	−1	−2	−3	−4	($\bar{d}$)		
O	3	10	13	13	7	2	1	1	0	1.48	1.47	7.12**
A_2	1	2	9	14	15	7	1	1	0	.60	1.33	3.17**
A_1	0	1	2	8	24	11	4	0	0	.08	1.03	.55
B	0	0	1	2	15	18	10	3	1	.94	1.13	5.88**
AB	0	0	1	2	21	16	5	4	1	.76	1.15	4.66**

** significance at the .01 level.

of the differences in the specificities of the anti-H_c and the anti-H_u reagents within blood groups and among different blood groups.

The first test is carried out by using the statistic "t", where $t=(\bar{d}-d)/s_{\bar{d}}$, and assuming that the two reagents have similar titers when tested with the same saliva. In the above formula $\bar{d}$ is the mean difference between the two reagents within groups; d is the expected difference, which under the hypothesis should be zero, and $s_{\bar{d}}$ is the standard error of the mean difference, which is found by $s_{\bar{d}}=s/\sqrt{n}$, where s stands for standard deviation. The results of the "t" tests give evidence for rejecting the hypothesis of homogeneity (significance at the 1 % level), between the two reagents for the A_2, O, the B and the AB salivas. On the other hand, their differences were not significant within the A_1 saliva group. More specifically, the potencies of the H_c and H_u are similar in the salivas of group A_1. The H_u potency is seen to be significantly higher in A_2 and O, whereas H_c is significantly more potent in the salivas of groups B and AB. The frequencies observed in Table III, have been arranged in the form of histograms in Figure 3.

TABLE IV

Analysis of Variance of the Differences in the Inhibition Titers of Anti-H_c and Anti-H_u.

Source	d.f.	s.sq.	m.sq.
Total	249	574	–
Between groups	4	200	50.00
Within groups	245	374	1.53

$$\text{"F"}=\frac{50.00}{1.53}=32.62^{**}, \text{ d.f. } 4, 245.$$

** significance at the .01 level.

To test whether the differences among the various blood groups are due to certain peculiarities of the reagents involved or due to chance variations, an analysis of variance was performed (Table IV). The "F" test is highly significant (at the 1 % level), which gives evidence that the differences between the various blood groups are not due to chance, but that the activity of the two anti-H reagents actually varies from group to group.

It is reasonable to expect that the threshold effect observed in the activity of anti-H_c would have decreased the actual titer of the saliva. That is, a specimen scored 7, with an agglutination reading of 3, might have been scored, in the absence of the threshold phenomenon, 8 or 9 with agglutination readings of 2 or 1. This effect, however, does not alter the fact that there is variability between the extracts; while the threshold increases the variation between the inhibition titers of the two anti-H reagents in the salivas of O and A_2 individuals, it tends to decrease it in the salivas of B and AB blood groups.

TABLE V

Inhibition Tests for Complimentarity of Anti-H Reagents Extracted from Three Different Seeds.

Neutralized reagent and dilution	Added reagent and dilution	Saliva Dilutions 1:6	1:7
Anti-H_c (1:4)	none	0	+
Anti-H_c (1:4)	Anti-H_c (1:4)	4	
Anti-H_c (1:4)	Anti-H_u (1:2)	0	
Anti-H_c (1:4)	Anti-H_t (1:16)	2	
Anti-H_u (1:2)	none	0	±
Anti-H_u (1:2)	Anti-H_u (1:2)	+	
Anti-H_u (1:2)	Anti-H_c (1:4)	0	
Anti-H_u (1:2)	Anti-H_t (1:16)	0	
Anti-H_t (1:16)	none	0	±
Anti-H_t (1:16)	Anti-H_t (1:16)	+	
Anti-H_t (1:16)	Anti-H_c (1:4)	1	
Anti-H_t (1:16)	Anti-H_u (1:2)	0	

Finally, a neutralization test was carried out. In this experiment a third anti-H reagent was included (*Tetragonolobus purpureus*), anti-H_t. The dilutions of ¼ for anti-H_c, ½ for anti-H_u and $^1/_{16}$ for anti-H_t were selected, since, at these dilutions, all three were inhibited by the type O saliva at $^1/_6$ dilution, but all three demonstrated only partial inhibition when the saliva was diluted to $^1/_7$ (Table V). Four tubes of each reagent were neutralized with the same saliva at a dilution of $^1/_6$. After neutralization, the first tubes of each reagent

served as the negative controls. In the second tube of each set, an additional drop of the same anti-H extract was added. In the remaining two tubes, one drop of either of the other two reagents was placed. The results of the neutralization tests recorded in Table IV support the statistical findings, that the anti-H_c and anti-H_u are not testing for the same substance.

Note that neutralization with anti-H_c is apparently complete as far as the recovery of additional anti-H_c is concerned but is not complete as far as the added anti-H_u is concerned. (We say "apparently" complete, for it is not certain how the threshold effect evident in the use of *Cytisus* may affect such a neutralization experiment.) Neutralization with anti-H_u, on the other hand, inactivates the saliva's further neutralizing capacity only towards anti-H_u. The anti-H_t behaves, in all cases, as the anti-H_c. The reactions noted in the table support the statistical findings that anti-H_c and anti-H_u each sah a unique anti-H specificity.

Preliminary experiments with rabbit immune sera have demonstrated at least one antigenic difference between the two extracts. Rabbit anti-*Cytisus* inhibits the agglutination activity of *Cytisus* extract toward O red blood cells but does not affect the activity of *Ulex* extract. Rabbit anti-*Ulex*, however, inhibits both extracts. The relation of the agglutination inhibiting antibody to an antibody capable of inhibiting the saliva neutralizing capacity of the extracts awaits further investigation.

Summary

Test of two commonly used anti-H reagents (extracts from *Cytisus sessilifolius* and *Ulex europaeus*) demonstrated that group O secretors have the highest titer of H substance, with A_2, A_1, B, and AB following in that order. A comparison between the two extracts indicated that saliva belonging to persons of group A_1 have about the same amount of both H_u and H_c (substances inhibiting the *Ulex* and *Cytisus* extracts respectively), A_2 and O groups have a much higher titer of H_u substance than they do H_c, and B and AB groups demonstrate exactly the opposite qualities, with higher H_c than H_u titers. The most plausible explanation of the data seems to be that at least two different types of anti-H specifities are involved (anti-H_c and anti-H_u); these extracts, then, differ not only quantitatively, but qualitatively as well.

Résumé

Les test pratiqués avec deux réactifs anti-H utilisés habituellement (extraits du Cytisus sessilifolius et de l'Ulex europaeus) ont démontré que les secréteurs du groupe O possèdent le plus de substances H et ensuite, par ordre décroissant, les secréteurs des groupes A_2, A_1, B et AB.

Une comparaison établie entre les deux extraits a démontré que la salive des personnes de groupe A_1 a la même quantité de substances H_c et H_u (substances inhibant les extraits de Cytisus sessilifolius et d'Ulex europaeus respectivement), que la salive des personnes de groupe A_2 et O ont une contenance plus grande en substance H_u, que la salive des personnes de groupe B et AB montre des propriétés exactement contraires avec une contenance plus forte en H_c qu'en H_u. L'explication la plus plausible de ces résultats semble être le fait qu'il y a au moins deux spécificités différentes de l'anti-H (l'anti-H_c et l'anti-H_u). Ces extraits se différencient non seulement au point de vue quantitatif mais également au point de vue qualitatif.

Zusammenfassung

Untersuchungen mit zwei gebräuchlichen Anti-H-Reagentien (Extrakte von Cytisus sessilifolius und Ulex europaeus) zeigten, daß Sekretoren der Gruppe O die höchsten H-Substanz-Titer aufweisen. Bei Sekretoren der Gruppen A_2, A_1, B und AB sind die Titer niedriger, wobei der H-Substanz-Gehalt von den A_2- zu den AB-Individuen progressiv abnimmt. Vergleichsuntersuchungen mit den beiden Extrakten zeigten, daß der Speichel von A_1-Personen etwa gleichviel H_u- und H_c-Substanz enthält (H_u hemmt Ulex; H_c hemmt Cytisus). Der Speichel von A_2 und O-Sekretoren enthält wesentlich mehr H_u- als H_c-Substanz. Der Speichel von B- und AB-Sekretoren enthält umgekehrt mehr H_c- als H_u-Substanz. Die einfachste Erklärung dieser Befunde besteht in der Annhame, daß die Extrakte Anti-H-«Antikörper» mit unterschiedlicher Spezifität enthalten. Die unterschiedliche Wirkung der Extrakte beruht somit nicht bloß auf quantitativen, sondern auf qualitativen Differenzen der aktiven Komponenten.

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Authors' address: Dr. Ch. C. Plato and Dr. H. Gershowitz, University of Michigan Medical School, Department of Human Genetics, *Ann Arbor*, Mich. (USA)