

ON THE NATURE OF A₁ AND A₂ ANTIGENIC DIFFERENCES: THE ROLE OF GLYCOPROTEIN AND GLYCOLIPID AGGLUTINOGENIC GLYCOTOPES IN THEIR FORMATION

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We used absorption of monoclonal reagents of Workshop IV (anti-A₁ 2-24, 2-25, 2-26, 2-27; anti-A 2-4, 2-10, 2-28; anti-H 2-71, 2-74) and anti-A_{hp} protectin by A₁ and A₂ erythrocytes, as well as inhibition of the above reagents and protectin by glycoconjugates received by enzymatic treatment of membranes, chloroform-methanol method and column anion-exchange chromatography. Unlike anti-A, anti-A₁ reagents poorly reacted with glycolipid conjugates (A_{lp}), were stronger inhibited by glycoprotein ones of the alkaline type (A_{pr-0'}, pH zone of 7,9-7,65) and significantly poorer reacted with the acidic type of glycoprotein conjugates (A_{pr-1'}, A_{pr-2'}, pH zone of 7,1-6,5). From A₂ membranes, besides A_{lp}, A_{pr-1} zone revealed glycoconjugates with H specificity, while in the acidic zone A_{pr-2} were found. The antigenic spectrum of A₁ erythrocytes was represented more completely.

Introduction

On the basis of the previously made investigations on different specificity of ABH glycoconjugates of the glycoprotein and glycolipid origin demonstrated by some MAB's [1], it seemed interesting to study the nature of A₁ and A₂ antigenic differences from this point of view. The investigation could include comparison of the absorption ability of A₁, A₂ erythrocytes with respect to anti-A₁, anti-A₂ MAB's and anti-A_{hp} with the study of the inhibitory ability of A glycoconjugates of the glycoprotein and glycolipid type, as well as in the combination of absorption and inhibition. The use of different test erythrocytes here could improve selectivity of the reactions and facilitate analysis of the results in order to reveal a possible role of different types of glycoprotein and glycolipid agglutinogenic epitopes in the formation of A₁ and A₂ differences.

Materials and methods

Monoclonal antibodies (MAB's) were received for testing by the programme of the 2nd section of the IV International Workshop on monoclonal antibodies and antigens of red blood cells (Paris, 2001). Among the used MAB's there were anti-A₁ 2-24, 2-25, 2-26, 2-27, 2-4, 2-28, 2-10, anti-H 2-71, and 2-74.

An anti-A_{hp} preparation, a powder of the albumin gland of *Helix pomatia*, containing anti-A_{hp} for preparation of anti-A_{hp} solution in physiological saline, was received from the Institute of Forensic Medicine (Berlin, Prof. Otto Prokop).

Antigens. Isolation of glycolipids from erythrocyte membranes was made by the chloroform-methanol method [2], and that of glycoprotein fragments using erythrocyte treatment with 1 % solution of trypsin (Spofa, Slovakia) under the conditions described before [1]. The subsequent isolation and cleaning of glycoconjugates were made on DEAE cellulose (Reanal, Hungary) with elution in the NaCl gradient and decreasing pH, and on DEAE sephadex A-25 (Farmacia, Fine Chemicals, Sweden).

The exit fractions were assessed photometrically (under 205 nm, 280 nm and full UV spectrum) on SP-26 spectrophotometer (Specord) and serologically.

Serological studies

The reaction of hemagglutination was carried out in 96 U-bottom plates by the standard technique with the use of O, A₁, A₂, A₂B erythrocytes [3] under the visual (+) and microscopic (+m) control.

The score was calculated according to Marsh [4] from dilution results as indicated below:

4+	12 pt
3+	10 pt
2+	8 pt
1+	5 pt
+m	3 pt
+m	1 pt
–	0 pt

The reaction of inhibition of MAB's and anti-A_{hp} was performed with regard for a decrease of their agglutinating titre in 1 % solution of albumin:

after their 60-minute contact with an antigenic substrate (glycoconjugates), followed by a rerolling and 60-minute exposure to a suspension of test erythrocytes.

The reaction of absorption was performed under the room temperature with the ratio of antibodies/albumin/15% erythrocyte suspension = 1:2:1, 60-minute exposure, centrifugation and titration of the supernatant fluid according to the standard technique with test erythrocytes. In all the variants of the experiments, not less than 10 sessions with different samples of corresponding erythrocytes were made.

Results

A. Absorption of MAB's by erythrocytes

As it turned out, both MAB 2-24 and 2-10, as well as anti- A_{hp} sharply reduced their agglutinogenic activity after their absorption by A_1 erythrocytes (Fig. 1).

Another picture was observed after absorption by A_2 erythrocytes (with the same A_1 test erythrocytes). While the standard MAB 2-10 decreased the score from 41,4 to 10, an anti- A_1 MAB did it from 28,3 to 18,5, anti- A_{hp} from 37,7 to 22,2. Thus, as it should be expected, anti- A_1 MAB 2-24 reduced its activity to the lowest degree. The anti- A_{hel} reagent also decreased its activity significantly less than after absorption by A_1 erythrocytes. But the standard polypotent anti-A 2-10 was absorbed by the both types of erythrocytes actually with the same intensity.

The use of A_2 as test erythrocytes showed (fig. 1) that the agglutinating ability of these nonabsorbed reagents, judging by the score, was slightly lower in comparison with A_1 erythrocytes, particularly in MAB 2-24 that significantly decreased its titre (from 28,3 to 21), only under the condition of taking into account the reaction of agglutination under microscope. But the absorption by A_1 erythrocytes deprived this MAB of its ability to react with A_2 erythrocytes at all. Here, if absorption by A_1 erythrocytes eliminated the agglutinating ability of the MAB with A_2 erythrocytes, the absorption by A_2 erythrocytes resulted in a little reduction of its ability to react with A_1 (and A_2 as well) erythrocytes.

This peculiarity was manifested for other anti-A reagents too, but to a less degree, particularly for MAB 2-10, thereby making it possible to regard these monoclonal antibodies as significantly different.

Even more sharply the above fact was manifested when for absorption and testing A_2B erythrocytes were used. MAB 2-24, even nonabsorbed, al-

most did not react with A_2B erythrocytes (score= 6). After absorption by A_1 erythrocytes all the reagents almost lost their ability to react with A_2B erythrocytes (except, partly, anti- A_{hp}). But after absorption by A_2B erythrocytes it was only MAB 2-10 that significantly reduced its activity.

The above peculiarities in the absorption rate of anti-A reagents were clearly manifested when taking into account the titre (titre Ig) too, a small absorption rate by A_2 erythrocytes (with A_1 test erythrocytes) for an anti- A_1 MAB (2-24) being manifested even more pronouncedly.

It is difficult to explain the described difference in the absorption rate of the taken anti-A reagents only by quantitative antigenic differences. They can be explained by qualitative differences, but only under the condition that the antigenic spectrum of A_1 erythrocytes overlaps A_2 (all the more A_2B), i.e. as if A_2 and especially A_2B were "lacking", did not have enough expression of one of the types of A antigenic markers. It is in this variant that there can exist the revealed "universality" of the absorbing effect of A_1 erythrocytes on all reagents and the expressed absorption of anti-A MAB 2-10 by A_2 erythrocytes – under absence of absorption of anti- A_1 MAB 2-24 by these erythrocytes.

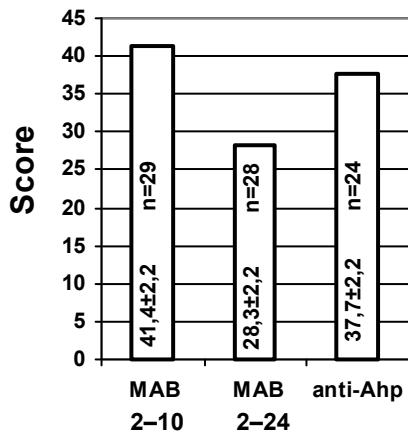
B. Inhibition of MAB's by glycoconjugates

We made an attempt to analyse this problem using A glycoconjugates of two types – obtained from the glycolipid fraction of erythrocyte membranes and from the glycoprotein fraction – after the enzymatic treatment of erythrocytes. It goes without saying that the enzymatic treatment with removal of glycoprotein receptors preceded the chloroform-methanol extraction of glycolipids from the membranes. The subsequent multiple ion-exchange chromatography contributed to separation of these two types of glycoconjugates too.

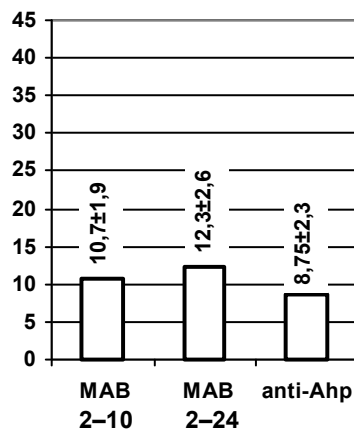
As it turned out, the used anti-A reagents were differently inhibited by glycoconjugates of the protein and lipid origin. While MAB 2-24 and anti- A_{hp} were inhibited to a greater degree by A_{pr} glycoconjugates, the standard MAB 2-10 was markedly inhibited by the both types of glycoconjugates (fig.2). But inhibition by glycoconjugates of the lipid origin (A_{lp}) for MAB 2-24, on the contrary, was manifested weaker, but in anti- A_{hp} , which can be used as an anti- A_1 reagent, it proved to have a particularly poor manifestation. On the whole for glycoconjugates of the lipid origin it resembled the pattern of absorption by A_2 erythrocytes (fig. 1).

Thus, MAB 2-24 and anti- A_{hp} manifested themselves as anti- A_{pr} reagents which reacted (were inhibited) to a greater degree with protein glycotopes

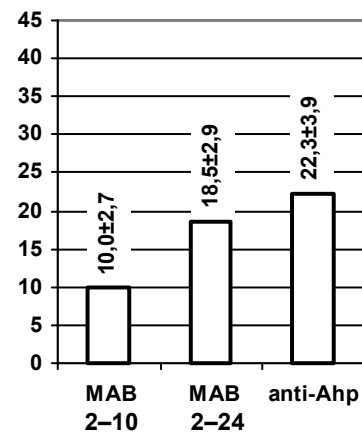
A₁ test erythrocytes



Reagents are not absorbed

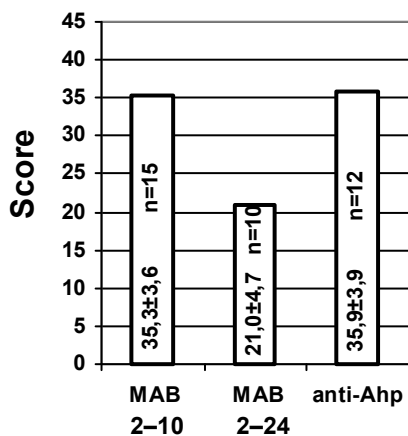


Absorbed by A₁ erythrocytes

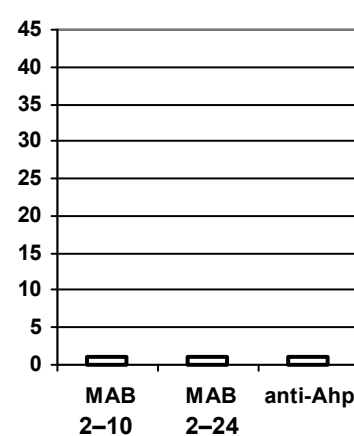


Absorbed by A₂ erythrocytes

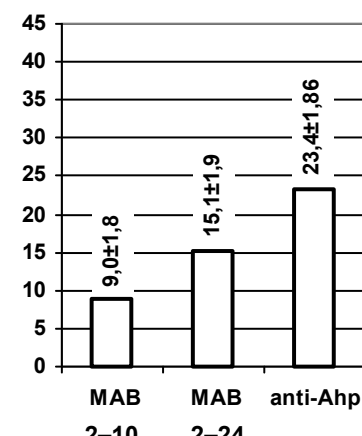
A₂ test erythrocytes



Reagents are not absorbed



Absorbed by A₁ erythrocytes



Absorbed by A₂ erythrocytes

Figure 1.
Absorption of anti-A reagent by A₁ and A₂ erythrocytes

(A_{pr} glycoconjugates) and poorly reacted with A_{lp} glycolipid. But as for MAB 2-10, it markedly reacted with the both types of glycoconjugates.

The selective ability of anti-A₁ reagents, especially MAB 2-24, to be inhibited by glycoconjugates of the protein origin was also clearly revealed in experiments with the preliminary absorption by A₂ erythrocytes and the subsequent testing for ability to be inhibited by the both types of glycoconjugates. MAB 2-24, which was almost not absorbed by A₂ erythrocytes, was poorly inhibited by A_{lp} too, but sharply reduced its agglutination ability after its contact with A_{pr}. That is, absorption by A₂ erythrocytes even increased selectivity in the reaction of this anti-A₁ MAB with A_{pr}.

The question arose: how regular is the peculiarity of interaction of this anti-A₁ reagent with glycoconjugates of the protein and lipid origin, or do they characterize only MAB 2-24?

On our request, Dr. T.N. Nikolaeva from the Russian Institute of Haematology and Transfusiology (Haematological Scientific Centre of the Academy of Medical Sciences of Russia) kindly sent three other MAB's with anti-A₁ specificity presented for Workshop IV (2-25, 2-26, and 2-27).

As it was expected, the titre of these MAB's with A₁ test erythrocytes turned out to be higher (1:512) than with A₂ erythrocytes (1:8-1:64), and even the manifestation rate of the reaction was different: agglutination with A₂ could be observed only under a microscope.

Naturally, particularly interesting was the ability of these MAB's to react with glycoconjugates of the glycolipid and glycoprotein origin. Taking into consideration the data obtained before, we checked this ability with the use of A₁ and A₂ test erythrocytes.

As it was revealed, under indication on A₂ erythrocytes no inhibition of MAB's 2-25, 2-26 and 2-27

A₁ test erythrocytes

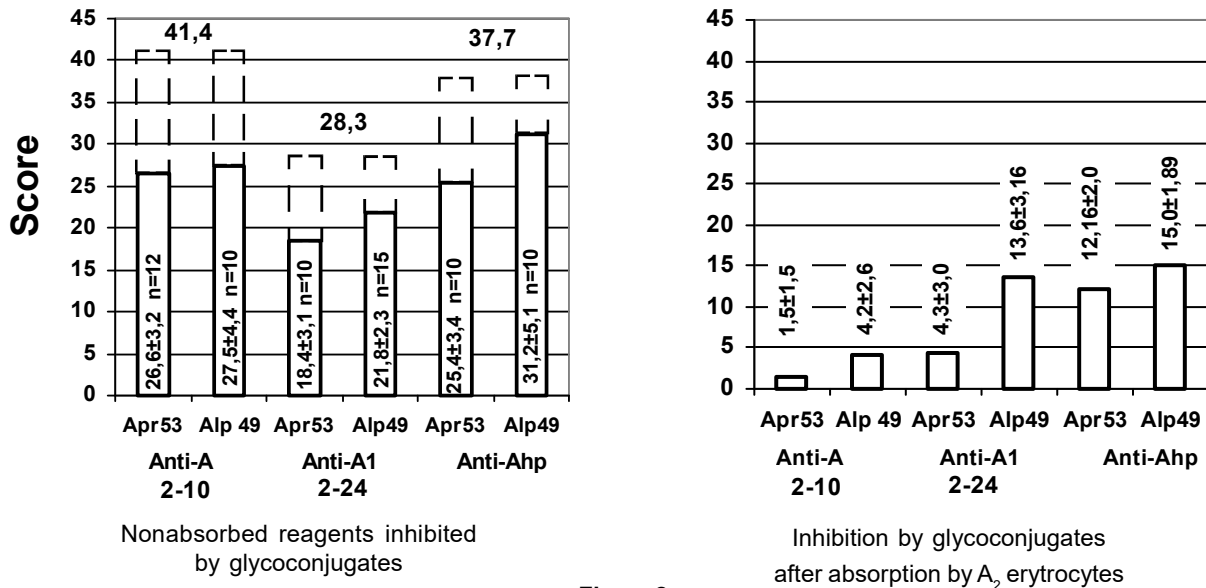


Figure 2.

Inhibition of anti-A reagents by glycoconjugates of two types (A_{pr} and A_{ip}) and in combination with absorption by erythrocytes

by A_{pr} glycoconjugates was manifested, but it was well expressed with A₁ test erythrocytes giving the titre drop by 2-3 grades.

As for glycoconjugates of the lipid origin, owing to A₂ test erythrocytes a contact with A_{ip} 37' preparation resulted in a decrease of the titre in MAB's 2-25 and 2-26 by 3-4 grades, and in 2-27 by 2 grades. But with testing on A₁ erythrocytes a decrease of A_{ip} by inhibition of the MAB titre was less expressed – 1 grade – and manifested only in some sessions.

Thus, anyway a poor ability of anti-A₁ MAB's to agglutinate A₂ erythrocytes almost absolutely disappeared after a contact with A glycoconjugates of the lipid origin of A_{ip} 37 type.

But the same type of A_{ip} conjugates, as it turned out, actually did not inhibit the same MAB's, if A₁ test erythrocytes were used instead of A₂ ones.

The impression is gained that A₂ erythrocytes are characterized by predominance of A-glycolipid marking with whom an anti-A₁ MAB poorly reacts. On the other hand, these antibodies are well inhibited by A_{pr} glycoconjugates, but under the condition of testing on A₁ erythrocytes, thereby it enabling us to believe that in A₁ erythrocytes this glycoprotein agglutinogenic type of marking is one of the basic types, along with the glycolipid one.

C. Effect of treating erythrocytes with trypsin

The ability to "take off" antigenic receptors from the cell membrane with the help of enzymatic treatment, particularly with trypsin, attracted research-

ers. But for ABH system this task proved to be not so simple: it is impossible to do it under usual conditions. We managed to solve this problem gradually decreasing pH of the medium in the process of trypsinization down to 7.2 (removing MN determinants) and then to 6.8-6.6 in order to remove ABH glycoprotein receptors [1].

It turned out that such a treatment significantly changed properties of A₁ erythrocytes as indicator cells and decreased the titre of anti-A₁ antibodies. It was revealed with MAB 2-25 (the titre decrease from 1:128 to 1:32) and MAB 2-26 (from 1:128-1:64 to 1:32), while in MAB 2-24 the treatment of A₁ erythrocytes with trypsin decreased the titre from 1:2048-1024 to 1:64.

A significant decrease of their titre with trypsinized A₁ erythrocytes was also observed in some other MAB's that before had manifested their ability to be expressively inhibited by A glycoconjugates of the protein origin, e.g. 2-4 (from 1:256 to 1:32). But the more polypotent MAB 2-10 decreased its titre significantly (from 1:1024 to 1:512). By the way, in all the above MAB's (2-24, 2-25, 2-26 and 2-4) their previously well-expressed ability to be inhibited by A_{pr} glycoconjugates failed to be revealed in case of indication by A₁ trypsinized erythrocytes.

D. Differences in inhibiting ability of Apr glycoconjugates

However, it would be too simple to bring the nature of A₁ and A₂ differences to two components – the glycolipid and glycoprotein markers. As it was

shown by further researches, an obvious persuasiveness of the above data in favour of the advantage of A_1 erythrocytes over A_2 ones – in a larger expressiveness of the glycoprotein A-marking if compared with A_2 – nevertheless requires significant specifications, particularly caused by heterogeneity of A glycoproteins of the erythrocyte membrane. It is also indirectly indicated by D.J. Anstee's [5] data on two types of ABH glycoproteins of the membrane which are responsible for transportation of either the protein or glucose.

Following trypsinization of erythrocytes, we isolated A-glycoconjugates of both a more alkaline type, pH zone of 7,9-7,65 (A_{pr-0}), and a more acidic one, pH zone of 7,1-6,5 (A_{pr-1} , A_{pr-2}), from the glycoprotein fraction, the character of the inhibitory effect for different MAB's in these types of glycoconjugates being significantly different. It is obviously demonstrated by a table of checking the serological activity of fractions by several MAB's even in case of the primary purification of the glycoprotein fraction on DEAE sephadex (tab. 1).

Table 1
Inhibition of MAB's by fractions produced during ion-exchange chromatography of supernatants after trypsinization of A_1 erythrocytes and by glycoconjugates isolated from glycolipids of membranes (A_{ip-0}).

MAB's	A_{pr-0} fractions 12-26	A_{pr-1} fractions 27-40	fractions 44-58	A_{pr-2} fractions 59-67	Fractions 81-84	A_{ip-21} , A_{ip-56} , glycoconjugates
2-24 anti- A_1	Score 26/22 -4	Score 26/22 -4	Score 26/21 -5	Score 26/19 -7	Score 26/20 -6	Score n = 15 26,7/23,2 3,5
2-4 anti-A	18/15 -3	18/16 -2	18/12 -6	18/12 -6	18/12 -6	n = 12 31,8/29,7 -2,1
2-28 anti-A	33/31 -2	33/31 -2	33/18 -15	33/17 -16	33/17 -16	n = 17 28,2/25,7 -2,5
2-10 anti-A	48/46 -2	48/47 -1	48/35 -13	48/33 -15	48/34 -14	n = 14 44,7/40,4 -4,3

As it is shown by the above data, anti- A_1 MAB 2-24 possesses the highest ability to be inhibited by fractions of the alkaline zone (12-26 and 27-40) with a relatively small (like in 2-4) ability to be inhibited by a group of more acidic fractions (44-84). It should be noted that A_{pr-53} preparation used by us for inhibiting anti-A reagents (see above) was isolated from the group of acidic fractions. Maybe for this reason its ability to inhibit anti- A_1 MAB 2-24 was relatively small (before absorption by A_2 erythrocytes) and slightly exceeded the inhibiting ability of glycolipid conjugates of the alkaline zone (A_{ip-49}).

And, on the contrary, the more universally specific MAB 2-10 demonstrates the weakest reaction with fractions of the alkaline zone. This MAB also has a well-expressed ability to be inhibited by fractions of the acidic zone.

By the way, the glycoconjugates isolated from the acidic zone are the least investigated and scarcely recognized type of A marking. And, maybe, this ability to strongly react with A_{pr-2} explains the fact of presenting MAB 2-28 as anti- A_4 or the ability to react with A_3 erythrocytes in the titre of up to 1:256 revealed in MAB 2-10. We would also like to remember the fact of isolating nonagglutinogenic A-glycoconjugate of this type in a donor with 0 (I) group – A_{pr-30} preparation which significantly inhibited the agglutination activity of MAB BRIC 145 (9W2) [1].

By the way, we isolated this type of A_{pr-2} glycoprotein marker in a donor of A_2 group, though it was characterized by a relatively small activity (A_{pr-33} preparation), but failed to reveal agglutinogenic A_{pr-1} in that donor; we managed to isolate a glycoconjugate with H specificity (H_{pr-1} preparation) from the alkaline zone of erythrocytes in the A_2 donor, this glycoconjugate inhibiting (with a score decrease by 6 pt) anti-H 2-71 and anti-H 2-74 MAB's.

Thus, talking about a more expressed glycoprotein A-marking in A_1 erythrocytes, one should apparently mean, both, its more alkaline type (A_{pr-1}) and the acidic one (A_{pr-2}).

Conclusion

Even the very presence of the testing anti- A_1 MAB's could be regarded as a ponderable argument confirming existence of qualitative differences between A_1 and A_2 erythrocytes. It was also clearly manifested in the difference of the absorption rate between anti-A and anti- A_1 MAB's (and anti- A_{hp} too) by A_1 , A_2 , and particularly A_2B erythrocytes. At the same time, the results of absorption make it possible to believe that the antigenic spectrum of A_1 erythrocytes overlaps A_2 and, apparently, includes both A_1 and A_2 marking.

Analysis of this problem with the use of two types of glycoconjugates – of the glycolipid and glycoprotein nature – showed that while the more polypotent standard anti-A MAB 2-10 was markedly inhibited by the both types of glycoconjugates; anti-A₁ reagents – MAB 2-24 and anti-A_{hp} – expressively reacted with A_{pr} glycoconjugates and were poorly inhibited by A_{lp} glycoconjugates. The preliminary absorption of anti-A₁ MAB's by A₂ erythrocytes increased still more the selectivity and the inhibition rate of this MAB by glycoconjugates of the protein origin.

The use of other anti-A₁ MAB's (2-25, 2-26, 2-27) made it possible both to confirm a greater inhibition rate of anti-A₁ reagents by A_{pr} glycoconjugates and to additionally reveal a very important circumstance: this inhibition is not revealed in case of testing on A₂ erythrocytes. And, on the contrary, inhibition by A conjugates of the glycolipid origin was manifested on A₂ erythrocytes (as far as it was allowed by a low titre of anti-A₁ with A₂ cells).

Thus, anti-A reagents, which can cause a strong and visually observed reaction with A₁ erythrocytes and a significantly less strong reaction with A₂, are inhibited to a greater degree by glycoconjugates of the protein origin produced after trypsinization of A₁ erythrocytes. But it turned out that, in its turn, this trypsinization changed the agglutinogenic properties of A₁ test erythrocytes sharply decreasing the titre of anti-A₁ reagents, if trypsinized A₁ test erythrocytes were used, but the most important thing was that in case of testing by trypsinized A₁ erythrocytes the inhibiting property of A_{pr} glycoconjugates was not revealed any more.

The subsequent study with division of A_{pr} glycoconjugates into a more alkaline A_{pr-0} fraction and a more acidic A_{pr-1}, A_{pr-2} one made it possible to state that the above advantage of A₁ (in a greater expressiveness of the glycoprotein marking) should be attributed to both A_{pr-1} – a more alkaline type of A receptors connected with proteins and to a more acidic one (A_{pr-1}, A_{pr-2}).

Since, as it was shown by the studies, the glycoprotein marking of A_{pr-1} type in A₂ erythrocytes is

weakened and substituted by H_{pr-1}, while the glycolipid one of A_{lp-1} type is preserved, then A₁ erythrocytes which usually have all the types of A_{pr} and A_{lp} A receptors are naturally revealed by antibodies which well react with A_{pr-1} but almost do not react with A_{lp-1} and poorly react with A_{pr-2}. It was this selectivity that was demonstrated, as it turned out, by anti-A₁ reagents MAB 2-24, MAB 2-25, MAB 2-26, MAB 2-27 and, partly, by anti-A_{hp} protectin which is rather intricate by its serological properties [6]. Naturally, trypsinization of A₁ test erythrocytes deprived these anti-A₁ MAB's of their ability to manifest this selectivity. The ability of MAB 2-10 to be strongly inhibited by both A_{lp} and A_{pr-2} (with a poor inhibition by A_{pr-1}) and the simultaneous inability of this MAB to differentiate A₁ and A₂ erythrocytes only confirm the truth of the above considerations.

In conclusion, we would like to notice that despite the qualitative differences revealed between A₁ and A₂ antigenic characteristic of erythrocytes one cannot but note presence of some degree of cross reactivity which, by the way, is less pronounced in case of using A₂B erythrocytes. It gives us the ground not to exclude a certain role also played by quantitative differences which may be caused by a degree of low expression of A_{pr-0}, A_{pr-1} and A_{pr-2} agglutinogenic glycotopess in A₂ erythrocytes.

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