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Epitope Mapping of Human Alpha-Fetoprotein

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Abstract—The epitope structure of human alpha-fetoprotein (AFP) was studied using more than 50 monoclonal antibodies (MAB) to human AFP. These MAB obtained from various world laboratories of the TD-2 AFP Workshops of the International Society for Oncodevelopmental Biology and Medicine (ISOBM-1996-1998-2000) were analyzed by competitive immunoaffinity electrochromatography (IAE) on nitrocellulose membranes (NCM). Five types of interaction of the AFP–MAB complex with the MAB fixed on NCM were found: 1) complete neutralization; 2) partial neutralization; 3) unidirectional neutralization; 4) enhanced binding; 5) lack of interaction. By IAE, 51 MAB were found to recognize 23 different epitopes in the AFP molecule. Based on these findings, an epitope map of AFP was designed which consists of eight epitope clusters and eight individual epitopes. The epitope location is considered with respect to the conformational state of the AFP molecule. Possible causes of the five types of interaction found on neutralization are discussed.

Key words: α-fetoprotein, anti-α-fetoprotein monoclonal antibodies, α-fetoprotein epitopes, epitope mapping of α-fetoprotein, immunoaffinity electrochromatography

Alpha-fetoprotein (AFP) is a serum protein synthesized by embryonic liver and yolk sac. Its synthesis ceases before birth and restarts in the case of hepatocellular cancer and germ-cell tumors [1, 2]. It is a single-chain glycoprotein consisting of about 590 amino acids combined in three domains and of glycan connected to asparagine in position 232. AFP is a polyfunctional protein which can selectively bind polyunsaturated fatty acids (PUFA) and estrogens, suppress various immune reactions, and, possibly, transfer PUFA into embryo cells [3]. These functions are thought to be mediated by different regions of the AFP molecule. Studies on antigenic determinants in the AFP molecule might be a useful approach to identification of these regions, and for this purpose it is reasonable to use monoclonal antibodies (MAB) strictly specific to different determinant groups (epitopes) in the AFP molecule. We mapped epitopes of human AFP earlier using various numbers of anti-AFP MAB: 6 [4], 12 [5], 24 [6], 30 [7], and 43 MAB [8].

In the present work, epitopes of human AFP were determined in the framework of the AFP Workshops of the International Society for Oncodevelopmental Biology and Medicine (ISOBM, 1996-1998) using 51 MAB, and a map of epitope topography in the AFP molecule is proposed.

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MATERIALS AND METHODS

Fifty-one MAB obtained from various countries were used. Thirty MAB were obtained from the Working Unit TD-2 ISOBM-1996-1998 under codes 92-121 [9]. Eleven MAB and the MAB E3, G6, G5, and B9 were prepared in our laboratory (altogether fifteen MAB). Eight MAB (Y(1-6), S2, and S4) were obtained from the laboratory of Professor Nishi (Chair of Biochemistry, Hokaido University). Seven MAB (97/2, 97/3, 97/4, 97/6, 97/8, 97/9, 97/10) were obtained from the Laboratory of Clinical Biochemistry, State Institute of Sera (Copenhagen, Denmark). Two MAB (AF/5 and Hy34/23) were obtained from Wallac OY (Finland). All antibodies in this collection were immunoglobulin fractions containing 0.5-1 mg protein/ml.

AFP isolated from human umbilical cord blood by immunoaffinity chromatography was used as the antigen. In some experiments a commercial 20% serum (placental) albumin was used which contained 100-150 μg AFP per ml (Mechnikov Institute, Moscow, Russia).

A purified immunoglobulin fraction (P 0128, DAKO, Denmark) labeled with horseradish peroxidase (anti-AFP–HP) was used as polyclonal antibodies to AFP.

The MAB were compared by immunoaffinity electrochromatography (IAE) [10-12] following the scheme presented in Fig. 1.

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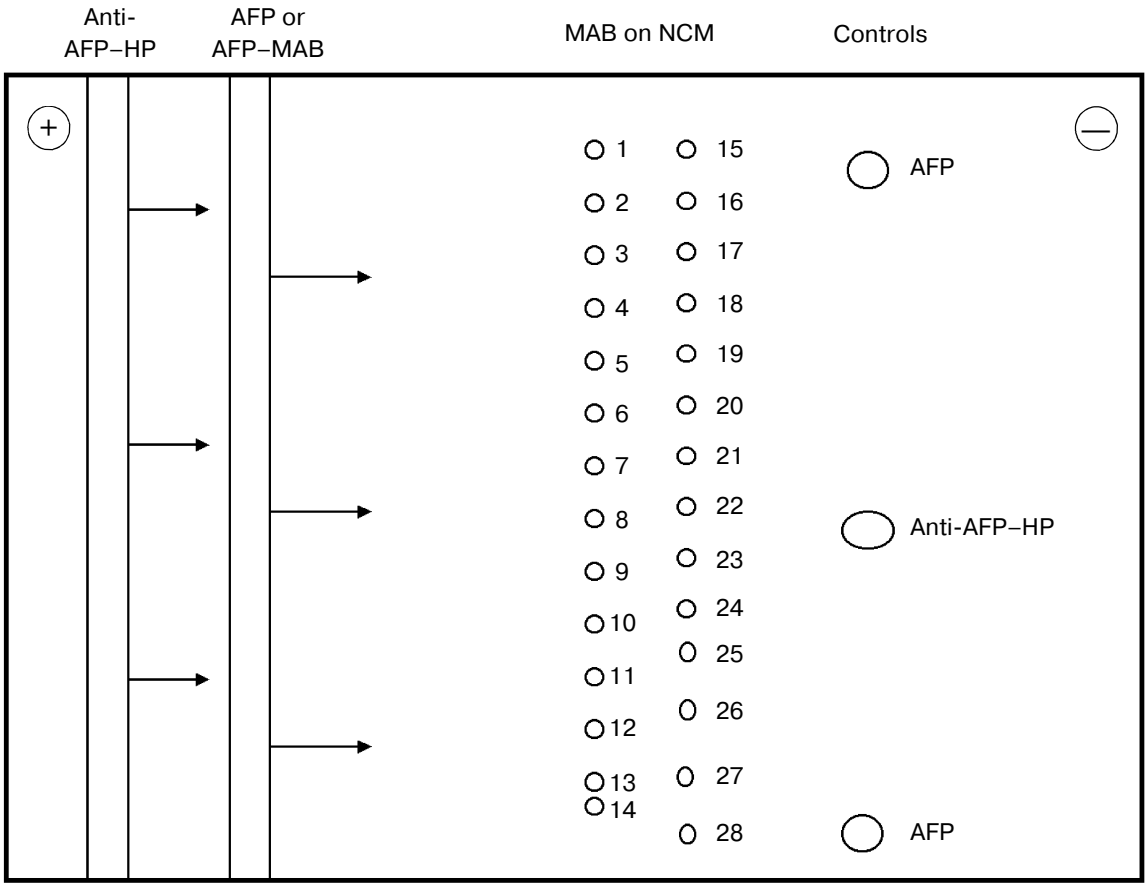


Fig. 1. Scheme for comparative analysis of anti-AFP MAB by immunoaffinity electrochromatography: 1-28) locations of anti-AFP MAB on NCM; anti-AFP–HP) conjugate of anti-AFP polyclonal antibodies with horseradish peroxidase. Experiment conditions: 1 μl of MAB (25-50 μg/ml) was applied onto NCM; 2 μl of AFP (100 μg/ml) were mixed with 25 μl of the analyzed MAB (1 mg/ml) and 73 μl of buffered saline.

All studied antibodies (1 μl, 20-50 μg/ml) were applied in chessboard order onto a strip of nitrocellulose membrane (NCM, Hybond C, Amersham, England). Control spots of AFP and of anti-AFP–HP were applied onto the cathode end of the NCM, and then the NCM was blocked with 0.5% casein (Sigma, USA) in buffered saline. Then the NCM was washed free of casein, and two folds (trenches) were made on its anode end 1-1.5 cm from the applied antibodies. The antigen to be analyzed or its mixture with MAB was placed in the trench nearest to the applied dots, and anti-AFP–HP was placed in the second trench. Electrophoresis was performed on the NCM strip in a system of two buffers: 0.06 M Tris-HCl (pH 6.7, the main buffer) and 0.012 M Tris-β-Ala (the closing buffer). Under these conditions, the rate of the cathode-directed endosmotic flow in the main buffer zone was higher than the rate of any protein with anode mobility, and this resulted in a successive transfer of proteins to the cathode, as if via a conveyor belt. When passing through the NCM-applied MAB, the antigen bound to them, and anti-AFP–HP which migrated after the antigen reacted with the bound antigen. IAE was carried at constant voltage (150 V) for 2-2.5 h. The reagent migration was visually monitored by the red color of vitamin B_{12} that was spotted before the electrophoresis at the anode edge of the trench with anti-AFP–HP. The electrophoresis was terminated after the spots of B_{12} had gone through the control spots. The reaction was detected by the staining developing during the reaction of peroxidase with H_2O_2 and diamino benzidine (DAB). The staining intensity, which corresponded to the binding degree, was estimated visually from 0 to +4.

An excess of any MAB added to the antigen solution was bound to the appropriate antigenic determinant of AFP and could not be fixed on MAB with the same specificity during the passage through the MAB panel, but it did not affect the AFP reaction with MAB of another specificity. In the first case the corresponding spot was not stained, whereas in the second case the staining was similar to the control (Fig. 2).

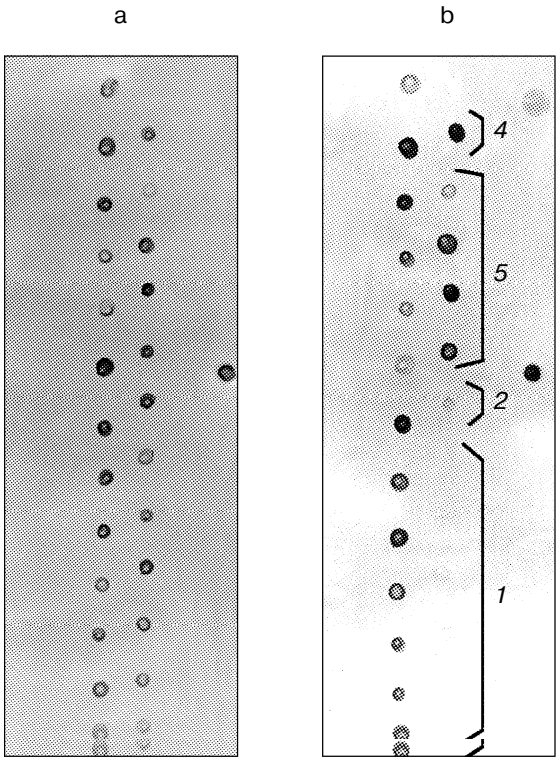


Fig. 2. Epitope analysis of anti-AFP MAB by immunoaffinity electrochromatography: a) binding of MAB applied onto NCM with the placental AFP (positive control); b) binding of MAB applied onto NCM with the placental AFP mixed with the analyzed MAB in solution (experiment). Reaction types: *1*) complete neutralization; *2*) partial neutralization; *3*) the reaction appeared similar to types *1* and *2* (not shown); *4*) enhanced binding; *5*) lack of neutralization.

RESULTS AND DISCUSSION

During the analysis of 51 MAB by inhibition of binding (Fig. 1), all MAB were used both as the inhibiting antibodies and, being applied onto NCM, as the immunosorbents for AFP–MAB complexes. Under conditions of 100-200-fold excess of MAB in the mixture with AFP, the following interaction types of the AFP–MAB complexes with antibodies applied onto NCM were recorded (Fig. 2): 1) complete mutual neutralization (inhibition, no staining), both MAB comparably suppressed the binding of AFP to its "partners", one on NCM and to the other in solution, and *vice versa*; 2) partial neutralization (incomplete inhibition, staining intensity varied from +1 to +3); in this case one of the MAB even in deliberate excess only partially decreased the binding of AFP to the other MAB on the solid phase; 3) unidirectional neutralization (one MAB inhibited the binding of AFP with the other MAB, but no inhibition occurred with the reverse setting of the reaction); 4) significantly increased binding of MAB to the antigen complexed with another antibody as compared to the free antigen; 5) complete lack of neutralization between the compared MAB. The findings are summarized in Table 1.

Table 1 presents results from anti-AFP MAB applied onto NCM and AFP-bound excess MAB in solution vertically and horizontally, respectively. Complete inhibition of reaction with the MAB applied onto NCM was a positive control for the same MAB excess with respect to its epitope. Thus, Table 1 shows the degree of similarity or difference between the MAB analyzed. Identical MAB mutually inhibiting the binding to AFP were combined into groups: group I, three MAB (93, 98, AF/5); group II, eight MAB (99, 110, 111, 117, 118, 119, 120, 97/10); group III, six MAB (95, 101, 105, 116, 121, 97/8); group IV, six MAB (97, 96, 112, 113, 107, G5); group V, five MAB (103, 104, 115, 94, 102); group VI, three MAB (B9, 97/6, Hy 34/23); group VII, two MAB (97/4, 97/3); group VIII, two MAB (Y5, Y2); group IX, two MAB (Y3, Y4).

The specificities of MAB in each of these groups were virtually the same, though there were some cross-reactions with other MAB and slight variations in their reactivities. Thus, based on the findings presented in Table 1, 37 of 51 MAB could be easily combined into compact distinct groups. There are nine such groups. The groups included various numbers of MAB with two to eight members. Fourteen of fifty-one MAB by their properties could not be *entirely* enlisted into any of the above-mentioned nine groups; therefore, they were presented as individual epitopes (X-XXIII). Thus, MAB 92 and 114, (epitopes XII and XIII) manifested identical properties with respect to group V but were different with respect to the groups III and IV. MAB 97/2 (epitope XV), MAB 97/9 (epitope XVI), and MAB 108 (epitope XVII) had the same properties with respect to groups VII and VIII, but varied with respect to groups I and IV. MAB 108 and 97/9 were the same in properties with respect to groups IV, VII, and VIII, but were different with respect to groups I and III. These MAB were cross-reactive but not identical. In the control, MAB S2 and S4 on NCM lacked the staining with AFP, but in the complex with the antigen they partially and unidirectionally suppressed the reaction of certain other MAB (the second and third reaction types) during competitive analysis. But since we had extremely small quantities of MAB S2 and S4, the findings on these MAB were separate, and they should be studied again.

MAB of group IV and also MAB 108 were different in the binding of various AFP preparations. Thus, they bound very weakly the AFP from human umbilical blood but showed a pronounced reaction with the AFP in the placental albumin preparation

Table 1 shows that the studied MAB recognized 23 epitopes in the human AFP molecule. Nine of them were recognized by large groups of identical MAB, and 14 epi-

Table 1. Epitope specificity by results of competitive analysis

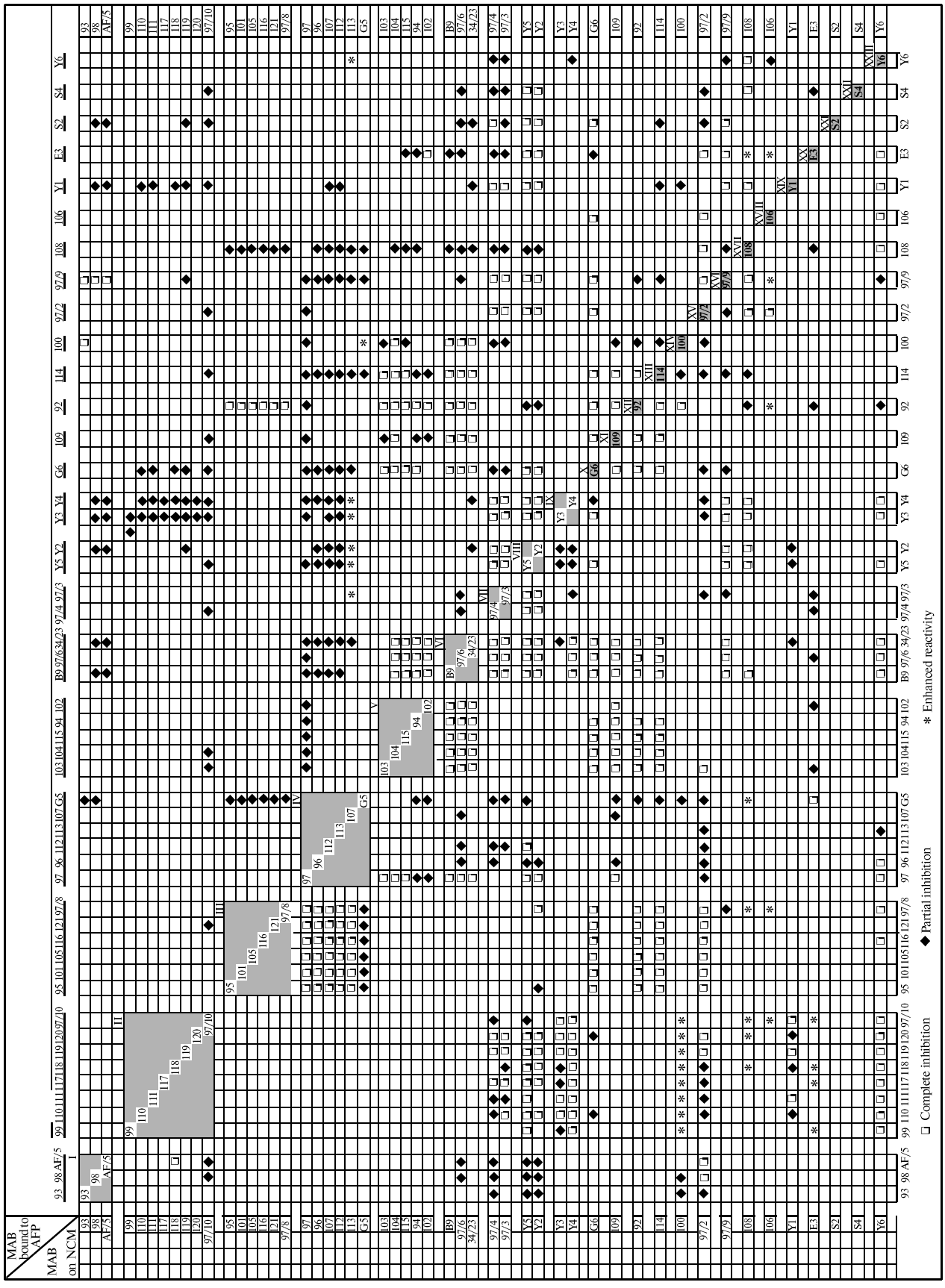


Table 2. Enhancement of the binding of the AFP–MAB complex with monoclonal antibodies fixed on NCM

MAB on NCM	Enhancing MAB (complexed with AFP)
100	group II
106	97/10, Y6, 97/9, 92, E3
108	118, 120, 97/10, G5, 97/8, E3
107	Y3, Y4, Y6, 97/3, Y2, Y5
E3	117, 118, 97/10, 99
G5	100

topes were recognized by individual MAB. Since AFP is a single polypeptide chain, these 23 epitopes are suggested to be unique, i.e., they are not repeated in the AFP molecule.

MAB responsible for enhancement of the AFP binding to MAB fixed on NCM (fourth type of reaction) were found also. Data on these MAB are presented in Table 2.

Tables 1 and 2 presenting the MAB and AFP interrelations were analyzed, and, as a result, a hypothetical map of epitope location in the AFP molecule has been suggested (Table 3). Table 3 is designed based on the findings presented in Tables 1 and 2, but the groups are combined by the principle "similar to similar", i.e., by the principle of the most compact "packing" of cross-reactive MAB from different groups with the overlapped epitope specificities, and this is suggested to depend on the epitope packing in the AFP molecule. For continuity, during the mapping the earlier names of epitope clusters A-E [7] were maintained. Twenty-three epitopes form in the AFP molecule 8 main clusters designated A, B, C, D, E, F, G, and H. Each cluster includes a number of MAB or groups with common epitope specificity. Based on significant similarity in properties of MAB in groups VII and VIII, they were combined into the same cluster (F). MAB of groups V, VI, X, XI, XII, and XIII were similarly combined into a cluster (B). It was also reasonable to combine epitopes XVI and XVII (97/9 and 108) into the common cluster G.

Clusters A, B, C, and E formed by MAB with high affinity for AFP [13] are the most compact and distinct, and cluster B is rather nonuniform in the properties of its constituent MAB because of their multiple point differences. Each cluster exists separately and autonomously but is also closely bound with one or more adjacent clusters either through the cross-reactive or through the common MAB. The cluster H shows cross-reactions with the E and F clusters and represents between them a kind of transition zone in concert with the individual epitopes Y1 and Y6. Similarly, cluster G connects the F and D clusters to each other. MAB G5 is a typical representative of both the D and A clusters, and MAB 92 is similarly a member of the A and B clusters. Cluster C is poorly connected with the adjacent cluster B through epitopes E3, 100, and 97/2, but through the same epitopes it is more "firmly" connected with cluster F. MAB 97/2 connects clusters C, B, A, D, and E among themselves, whereas MAB 97 plays the same role relative to clusters B, A, D, and G and partially for clusters F and H. Epitope Y6 connects clusters E, H, F, and B.

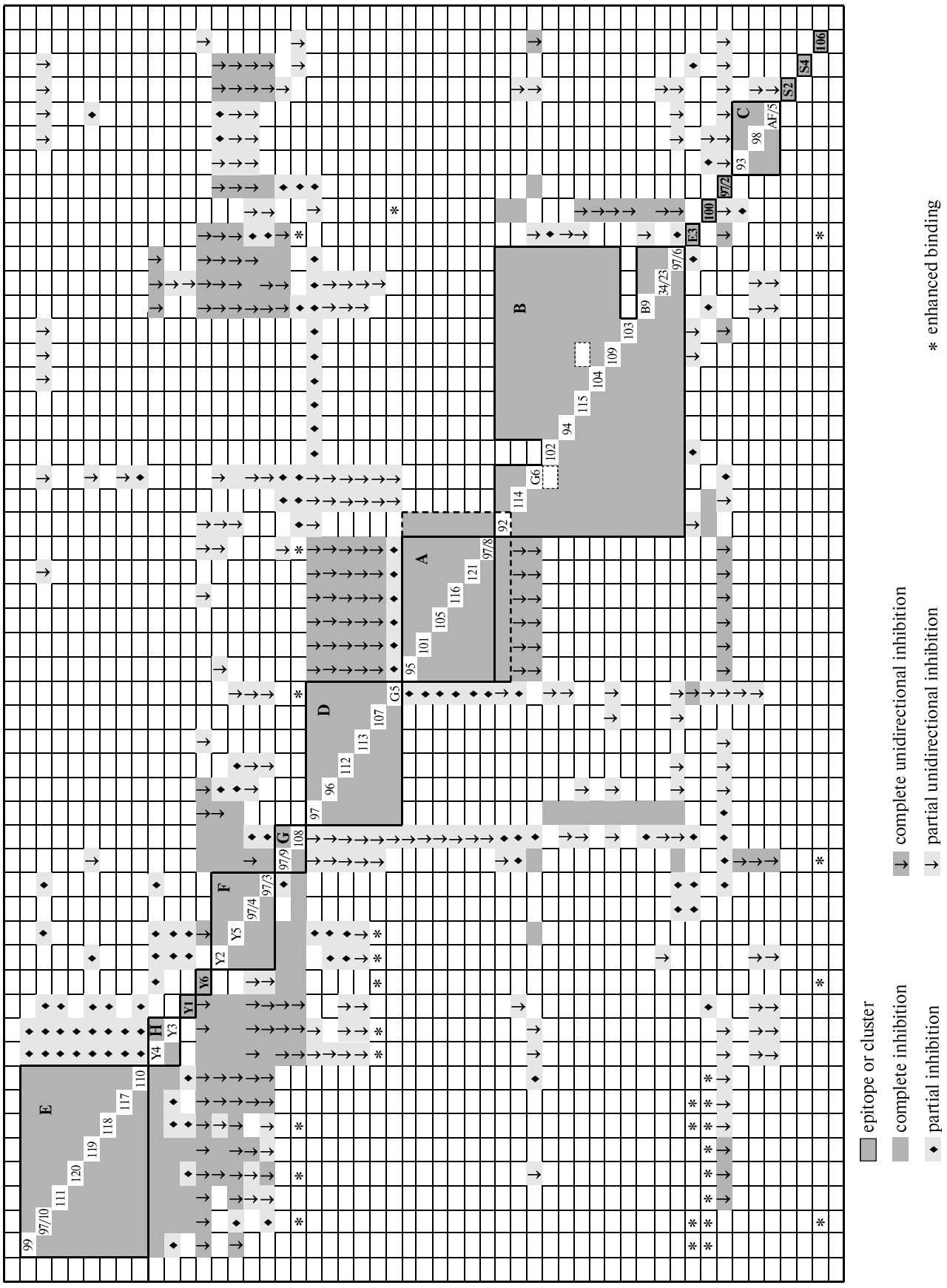
Clusters A, B, and E contain the greatest numbers of MAB; thus, they are suggested to be immunodominant epitopes of AFP because the MAB of these clusters were prepared in different laboratories [9]. Surprisingly, anti-epitope D MAB were prepared only in our laboratory [5]. And only with the anti-D MAB the epitope D expression in the AFP molecule was found in both "closed" and "open" form changeable from one to the other under various conditions [8, 14, 15]. Epitopes 106 and 108 display similar properties and with cluster D are markers of the conformational state of the molecule. The expression of these epitopes varies in the AFP from various sources [16, 17].

From unpublished data of Nishi and Christiansen et al., most of the epitopes were detected in domain III (clusters A, B, and D), and cluster E was located in domain I. This was also confirmed by separation of epitopes A, B, and D from epitope E by fragmentation of AFP [8]. Cluster C seems to be in domain II, and this is suggested to be connected with the carbohydrate region of the molecule because these MAB competed with lentyl-lectin for the binding to AFP [18].

One can see in Table 3 that there is no pronounced mutual effect between certain epitopes (B and E, C and A); however, most epitopes or their clusters show more or less pronounced cross-reactions. This finding, supplemented with data on location in the domains, indicates that the AFP epitopes are closely packed in the structure of the AFP molecule. Therefore, the complexing of AFP with some of 51 MAB in any case should affect its interactions with many MAB. A similar situation was found by RIA [19] of 14 MAB from five different epitopes. It is difficult to unequivocally explain the observed cross-reactions. The complete mutual neutralization of MAB (type 1) can be explained either by direction of the compared MAB to the same epitope or by prevention by the epitope space location of the simultaneous binding to the antigen of the two compared MAB.

The second type of interaction is suggested to be due either to only partial similarity in the properties of the epitopes involved (that prevents the reaction to be completed) or to expression of a certain epitope only in some AFP molecules [15]. In this case, the AFP molecules complexed with MAB are involved in the inhibition, whereas the non-complexed AFP molecules show a

Table 3. Map of epitope topography in the AFP molecule



weakened reaction with the corresponding MAB fixed on NCM, similarly to the case of the positive control. The third type of reaction seems to be associated with a partial spatial superposition and shielding of the epitopes that makes their shielded regions, as if on their "wrong side", inaccessible for the closely related antibodies. The boundary MAB binding different epitopes are probably directed to those regions of the AFP molecule that are spatially adjacent to each other not by covalent bonds, but by hydrogen or hydrophobic bonds, and this is responsible for the unique properties of such epitopes.

The enhancement of MAB reaction compared to the control (the fourth reaction type) can be seen based on the map. This reaction type seems to be inherent to those epitopes which are opened on removal of the shielding epitopes. Thus, if cluster E in the AFP molecule is blocked, the remaining part of the AFP can easily interact with MAB 100, E3, or 108, which were shielded by cluster E in the control. This is manifested visually by enhancement of the reaction, and this seems to indicate that the involved epitopes are disposed closely and that the shielding agent E is located on the external side of the molecule. It is likely that MAB manifested by enhanced reaction are directed to the internal epitopes that are shielded by their neighbors. Thus, blocking of the "boundary intermediate" epitopes Y6, 108, E3 or of a region of cluster E increases the interaction of MAB 106 with AFP, and this results in the appearance of epitope 106 earlier hidden by them, i.e., this epitope is located inside the molecule. However, the enhancement phenomenon can also be explained otherwise: anti-E MAB binding to epitope E results in such a strong modification of the AFP molecule that the whole conformation of the structure is changed. This uncovers the epitopes hidden inside the molecule, including those rather distant from epitope E, and they become accessible for the appropriate MAB. We have shown these two possibilities in experiments with peroxidase-labeled AFP: AFP–HP complexing with MAB 118 was associated with a sharply increased binding of AFP to MAB 108 applied on NCM (unpublished data).

In conclusion, it should be noted that this study using an increased number of MAB (from 30 to 51) has not revealed fundamentally new clusters, but has only refined and supplemented the picture of mutual interactions of epitopes by suggested transition zones between the epitopes. Also, the use of AFP from umbilical blood or from placental albumin had no significant influence on the epitope map.

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