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Determination of Different Antigenic Sites on the Adenovirus Hexon Using Monoclonal Antibodies

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With 8 Figures

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Summary

Eighteen mouse ascitic fluids containing monoclonal antibodies (MAbs) directed against crystallized hexon of adenovirus (AV) type 1 were used to map the antigenic structure of the capsomer in reciprocal competitive binding ELISA. With the help of peroxidase-labelled MAbs at least nine epitopes (epitope clusters) located on three distinct antigenic sites were identified on the hexon. Epitope on antigenic site I recognized by two MAbs could be the genus specific antigenic determinant based on the broad reactivity patterns of the MAbs. Epitopes on the antigenic site II recognized by fifteen MAbs could be divided into seven epitope clusters according to the competition patterns. Antigenic site III recognized by one MAb completely differs from the antigenic site I and on the basis of one-way blocking with all the MAbs specific for antigenic site II, should be also different from the latter one. The data suggest that the seven epitope clusters of antigenic site II contain partially overlapping epitopes and may be a part of a large single immunodominant antigenic region on AV 1 hexon as well as on hexons of heterologous types.

Introduction

Monoclonal antibodies have proven to be useful tools in determining the important antigenic regions of structural virus proteins. The introduction of the MAbs into the antigenic analysis of adenovirus hexon protein resulted the recognition that the capsomer has more complicated antigenic structure as was known earlier (5, 12, 13, 14, 18). Using a panel of MAbs produced against crystallized AV 1 hexon for the examination of purified heterologous

hexons it was demonstrated that on the surface of the hexon a large number of epitopes do exist, some of which are overlapping and characterized as interspecies specificities (2).

In the present paper, we report on the delineation of different antigenic sites and epitopes (epitopes clusters) of the adenovirus hexon by means of competitive binding ELISA.

Materials and Methods

Virus Strains, Purification of Hexons

Human adenovirus types 1, 2, 5, and 6 (subgenus C), 8, 9, 10, and 13 (subgenus D), 12 (subgenus A), 7 and 35 (subgenus B) were used in the experiments for determining the cross-reactivity of the MAbs. All these strains were propagated on HEp-2 cells, and the soluble hexon proteins of all virus types were separated and purified as described earlier (9, 11).

Hybridoma and Mouse Ascites Production

Spleen cells of Balb/c mice immunized with crystallized AV 1 hexon were fused with Sp 2/0 myeloma cells. Hybrid cells producing specific MAbs were inoculated i.p. into mice, and the developed ascites was sucked from the abdomen. The reactivity patterns of the MAbs were determined with 11 different hexon types using indirect ELISA (2).

Purification of Monoclonal IgG

Saturated ammonium sulphate was added to the ascitic fluids to a final concentration of 40 per cent. After centrifugation at 2000 × *g* for 20 minutes, the pellet was resuspended in 40 per cent saturated ammonium sulphate. After three times repeated centrifugation, the pellet was resuspended in distilled water and dialysed against PBS pH 7.2, at 4° C, overnight.

Peroxidase Labelling of Monoclonals

MAbs were dialysed against 0.15 M NaCl and labelled with horseradish peroxidase (REANAL, Budapest) according to the technique of AVREMEAS and TERNYNCK (1). Briefly, 10 mg peroxidase were dissolved in 0.2 ml phosphate buffer pH 6.8 containing 1 per cent glutaraldehyde, incubated at room temperature for 18 hours, and passed through a Sephadex G-25 column. Brown-coloured fractions were collected and added to 5 mg of MAbs. The mixture was stored at 4° C for 24 hours, and dialysed extensively against PBS. Before competitive-binding assays, each conjugate was titrated by direct ELISA, and used in the competition experiments in predetermined dilutions with a resulting absorbance of approx. 1.4, measured at 492 nm.

Competitive-binding ELISA

Polystyrene plates (Novogen) were coated with purified AV 1 hexon (250 ng in 50 µl/well) in PBS pH 7.2, overnight. Nonspecific binding was reduced to a minimum by the addition of PBS-Tween 20 containing 0.5 per cent BSA (Serva, Heidelberg) for 1 hour. Dilutions of unlabelled competitor MAbs causing 100 per cent inhibition of the own labelled probe were the starting dilutions (dilution factor 1), and serial two-fold dilutions of competitor MAbs were added, followed immediately by the predetermined dilution of the labelled MAbs. The plates were incubated for 90 minutes at 37° C, then washed and the bound peroxidase-labelled MAbs were detected by adding 50 µl of ortho-phenylenediamine (OPD). The reaction was stopped by the addition of 50 µl of 4 M H_2SO_4 per well. Optical

density was determined at 492 nm with a Titertek Multiskan photometer. Since the non-specific binding of individual conjugates varied considerably, binding to control antigen (BSA) was taken to represent background, and binding of each conjugate to viral antigen as maximum binding. This difference was taken to represent 100 per cent binding, and the relative inhibition by the competing first antibody was normalized to this scale in each individual test. Results were expressed as a percentage of competition, and rated as complete inhibition if the first three dilutions caused more than 70 per cent reduction of absorbance, partial inhibition if the reduction was between 60 to 40 per cent and results were considered negative if the reduction of absorbance was not more than 40 per cent (17).

On the other hand, competitive-binding ELISA was performed with a mixture of labelled MAbs (designated 1 A 3, 2 A 1, 2 A 6, and 2 B 2) and different concentrations of purified hexon types preincubated at 37° C for 2 hours (6). The wells were coated with AV 1 hexon (5 μg/ml) and the inhibiting ability of different hexon types was calculated as mentioned above.

Results

Differentiation of Antigenic Sites on the Adenovirus Hexon

Eighteen MAbs were used in unlabelled and peroxidase-labelled form to differentiate topographically distinct antigenic sites on AV 1 hexon. The terminology of YEWDELL and GERHARD (19) was used to define the epitopes and antigenic sites, i.e., an antigenic site consists of a cluster of epitopes, and the epitope is the combining site of a single MAb.

Three different antigenic sites could be separated on the basis of reciprocal competition experiments (Table 1). Epitope(s) of antigenic site I recognized by two MAbs is completely different from antigenic sites II and III, i.e., MAbs 1 A 3 and 2 C 2 cause complete inhibition of each other

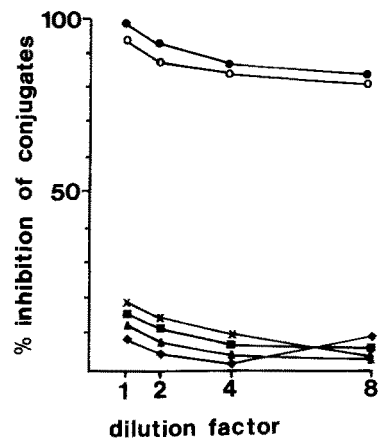

Fig. 1. Reciprocal competitive binding ELISA with the MAbs specific for antigenic sites I and III, and a representative of antigenic site II. All the unlabelled MAbs were used in a starting concentration which caused 100 per cent inhibition of the own peroxidase labelled form. ● 1 A 3/2 C 2; ○ 2 C 2/1 A 3; ■ 1 A 3/2 C 3; ▲ 2 C 3/1 A 3; ◆ 1 A 3/H 12; × H 12/1 A 3

Table 1. *Reciprocal competitive binding ELISA with 18 MAbs showing different competition patterns*

		1A3[a]	2C2	2C3	1D4	1A5	1D2	2B2	2C6	2C4	1B2	2A5	1A6	2B5	2C1	2D6	2A1	2A6	H12
I	1A3[b]	●	●	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	2C2	●	●	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
II/1	2C3	–	–	●	●	●	●	●	●	●	●	●	●	●	●	×	×	×	●
	1D4	–	–	●	●	●	●	●	●	●	●	●	●	●	●	×	×	×	●
	1A5	–	–	●	●	●	●	●	●	●	●	●	●	●	●	×	×	×	●
II/2	1D2	–	–	●	●	●	●	×	●	●	●	●	●	●	●	×	×	×	●
	2B2	–	–	●	●	●	×	●	●	●	●	●	●	●	●	×	×	×	●
II/3	2C6	–	–	●	●	●	●	●	●	×	●	●	●	●	●	×	×	×	●
	2C4	–	–	●	●	●	●	●	×	●	×	×	●	●	●	×	×	×	●
	1B2	–	–	●	●	●	●	●	●	×	●	●	●	●	●	×	×	×	●
	2A5	–	–	●	●	●	●	●	●	×	●	●	×	●	●	×	×	×	●
II/4	1A6	–	–	●	●	●	●	●	●	●	●	×	●	×	●	×	×	×	●
	2B5	–	–	●	●	●	●	●	●	●	●	●	×	●	×	×	×	×	●
	2C1	–	–	●	●	●	●	●	●	●	●	●	●	×	●	×	×	×	●
II/5	2D6	–	–	×	×	×	×	×	●	×	×	●	●	×	×	●	●	●	●
II/6	2A1	–	–	×	×	×	×	×	×	×	×	×	●	×	×	●	●	●	●
II/7	2A6	–	–	×	×	×	×	×	×	×	×	×	×	×	×	●	●	●	●
III	H12	–	–	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	●

[a] Peroxidase labelled MAbs
[b] Competitor MAbs

● Complete inhibition
× Partial inhibition

– No inhibition

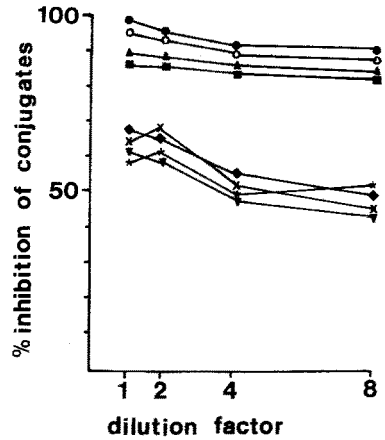

Fig. 2. Differentiation of antigenic site II and III on the basis of one-way blocking pattern using some representatives specific for different epitope clusters of antigenic site II, and the MAb H 12 specific for antigenic site III. ● 2 C 3/H 12; ○ 2 C 4/H 12; ▲ 2 D 6/H 12; ■ 2 A 6/H 12; ◆ H 12/2 C 3; × H 12/2 C 4; ▼ H 12/2 D 6; * H 12/2 A 6

recognizing probably the same epitope on the antigenic site I and these MAbs could not inhibit the binding of the labelled MAbs specific for antigenic sites II and III (Fig. 1). MAbs specific for antigenic site II did not inhibit the binding of the peroxidase labelled MAbs specific for antigenic site I, but could cause complete and partial inhibition of the binding of the MAbs specific for different epitope clusters of antigenic site II (Table 1). MAb H 12 could not inhibit the binding of the MAbs of antigenic site I (Fig. 1). This MAb partially inhibited the binding of the MAbs specific for antigenic site II, showing only a one-way blocking pattern (unidirectional blocking) (Fig. 2). As only reciprocal (bidirectional) complete blocking was taken to indicate identical antigenic sites, MAb H 12 probably recognizes a separate, third antigenic site (Table 1).

Overlapping Epitope Clusters on Antigenic Site II

Epitopes on the antigenic site II could be clustered into seven groups based on complete and partial reciprocal inhibition (Table 1). MAbs of epitope cluster II/1 completely inhibit the binding of the MAbs II/2 to II/4, and partially inhibit the binding of MAbs specific for epitope clusters II/5 to II/7 (Fig. 3). This kind of competition patterns could characterize two larger groups of closely related epitopes. One of them could be the epitopes clustered as II/1 to II/4, and the other characterized as epitopes II/5 to II/7. The differentiation of epitope cluster II/2 to II/4 is based on the different partial reciprocal inhibition of the MAbs grouped into these clusters. MAbs of epitope cluster II/2 completely inhibit the binding of MAbs specific for epitopes of II/1, II/3 and II/4 in reciprocal fashion, but they can inhibit each

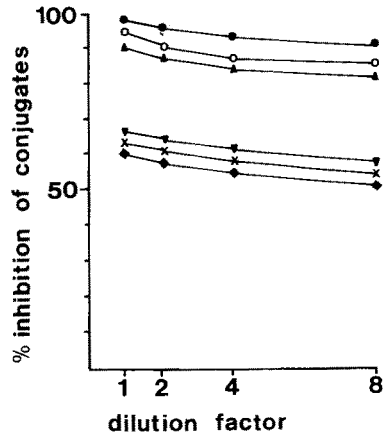

Fig. 3. Reciprocal competitive binding ELISA using MAbs representing epitope clusters of antigenic site II (Table 1). MAb specific for epitope cluster II/1 completely inhibits the binding of the representatives of epitope clusters II/2 to II/4, and partially inhibits the binding of the MAbs specific for epitope clusters II/5 to II/7. ● 2 C 3/1 D 2; ○ 2 C 3/2 C 6; ▲ 2 C 3/1 A 6; ▼ 2 C 3/2 D 6; × 2 C 3/2 A 1; ◆ 2 C 3/2 A 6

other partially (Fig. 4). MAbs of epitope cluster II/3 show another form of reciprocal competition, i.e., one of the MAbs (2 C 4) partially inhibits the binding of three other MAbs (2 C 6, 1 B 2 and 2 A 5), and the latter three MAbs completely inhibit each other (Fig. 5). MAbs specific for epitope cluster II/4 show similar inhibition patterns as those of epitope cluster II/3. It means, that one MAb of this group (2 B 5) could partially inhibit the binding of two other MAbs (1 A 6 and 2 C 1) showing reciprocal partial inhibition patterns, but the two latter MAbs completely inhibit the binding of each

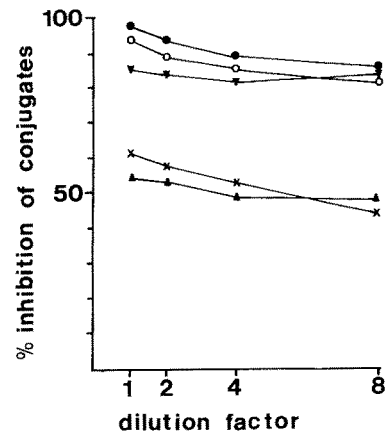

Fig. 4. Reciprocal competitive binding ELISA with the MAbs specific for epitope clusters II/2, and II/1, II/3, II/4. MAbs of epitope cluster II/2 inhibit partially each other in reciprocal fashion, and completely inhibit the binding of the MAbs specific for other epitope clusters. ● 1 D 2/2 C 3; ○ 1 D 2/2 C 6; ▼ 1 D 2/1 A 6; × 1 D 2/2 B 2; ▲ 2 B 2/1 D 2

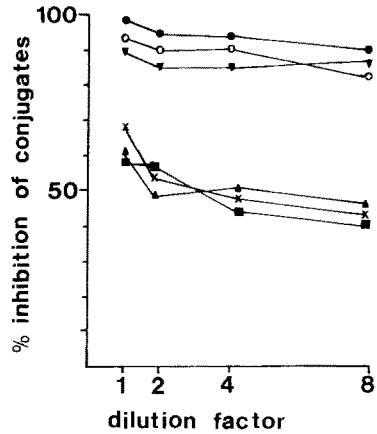

Fig. 5. MAbs of epitope cluster II/3 (1 B 2, 2 C 6, and 2 A 5) show reciprocal partial inhibition with the fourth member of this epitope cluster (MAb 2 C 4), but complete inhibition with each other. ● 2 C 6/1 B 2; ○ 2 A 5/2 C 6; ▼ 1 B 2/2 C 6; × 2 C 4/2 C 6; ▲ 2 C 4/1 B 2; ■ 2 C 4/2 A 5

other (Fig. 6). The differentiation of epitope clusters II/5 to II/7 is based on different complete and unidirectional blocking results. MAb specific for epitope cluster II/5 (2 D 6) could completely inhibit the binding of two MAbs of epitope cluster II/3 (MAbs 2 C 6 and 2 A 5), and of MAb 1 A 6 of epitope cluster II/4. MAb 2 A 1 (epitope cluster II/6) shows unidirectional complete inhibition of MAb 1 A 6 of epitope cluster II/4, while MAb 2 A 6 (epitope cluster II/7) shows reciprocal partial inhibition of all the MAbs clustered into II/1 to II/4 (Table 1). The three MAbs specific for epitope clusters II/5 to II/7 showed complete bidirectional inhibition of each other.

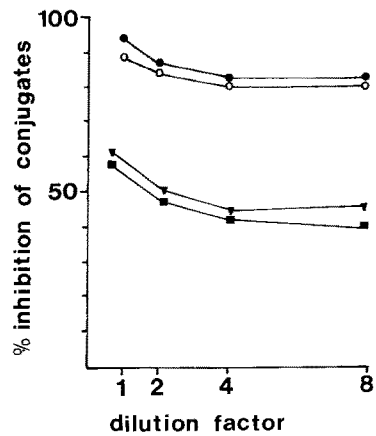

Fig. 6. Reciprocal competitive binding ELISA with MAbs specific for epitope clusters II/4. MAb 2 B 5 shows reciprocal partial inhibition with MAbs 1 A 6, and 2 C 1, but the latter ones inhibit each other completely. ● 1 A 6/2 C 1; ○ 2 C 1/1 A 6; ▼ 2 B 5/1 A 6; ■ 2 B 5/2 C 1

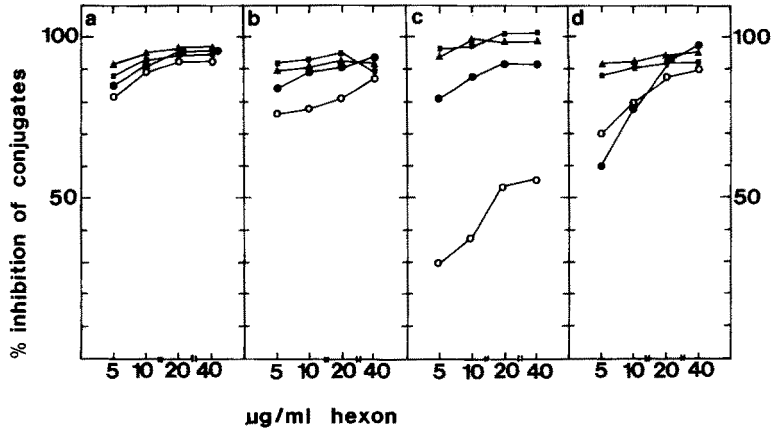

Fig. 7. Competitive binding ELISA with different hexon types of subgenus C. AV 1 hexon coated wells were incubated with an appropriate dilution of each conjugated MAb that had previously been incubated with type 1 *(a)*, type 2 *(b)*, type 5 *(c)* and type 6 *(d)* hexon. Peroxidase labelled MAbs: ● 1 A 3; ▲ 2 A 1; ■ 2 A 6; ○ 2 B 2

The Presence of Different Epitopes on Heterologous Hexons

For the determination of the presence of different AV 1 hexon related epitopes on the heterologous hexon types, four of the labelled MAbs (1 A 3, 2 A 1, 2 A 6, and 2 B 2) were mixed with serial dilutions (5 to 40 µg/ml) of different hexon types. After preincubation at 37° C 50 µl of each mixture was added to AV 1 hexon coated wells and the absorbances were determined both in the presence and in the absence of heterologous hexon types. Fig. 7 a–d shows the results with AV 1 hexon and with the heterologous

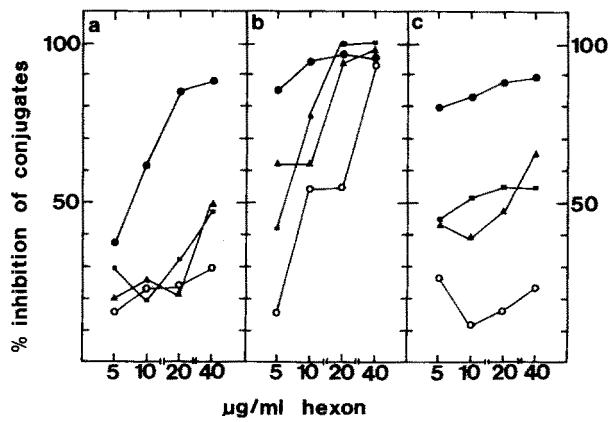

Fig. 8. Competitive binding ELISA with different hexon types of subgenus D. AV 1 coated wells were incubated with each conjugated MAbs that had previously been incubated with type 8 *(a)*, type 9 *(b)*, and type 10 *(c)* hexon. Peroxidase labelled MAbs: ● 1 A 3; ▲ 2 A 1; ■ 2 A 6; ○ 2 B 2

types of the same subgenus (types 2, 5, and 6). As can be seen, in the case of the MAbs 1 A 3, 2 A 1, and 2 A 6, each hexon types mixed with the MAbs causes nearly 100 per cent inhibition. The results with types 1, 2, and 6 were similar, when mixed MAb 2 B 2, while type 5 could not compete the binding of this MAb. The curves representing the competition with heterologous types of subgenus D (Fig. 8 a–c) only in the case of MAb 1 A 3 are similar to the types of subgenus C, and in high concentration of type 9 with MAbs 2 A 1, 2 A 6, and 2 B 2. Hexon types 8 and 10 could not cause complete inhibition even in high concentration.

Discussion

Eighteen MAbs were used in unlabelled and peroxidase-labelled form in reciprocal competitive binding ELISA to map the antigenic structure of the hexon. Each MAb was compared with all other MAbs and the inhibition patterns were characterized as complete or partial, bidirectional (reciprocal) or undirectional (one-way) inhibition (4).

At least three antigenic sites (I to III) were determined on the base of the results, and seven epitope clusters (epitopes) could be separated on the antigenic site II. The two MAbs (1 A 3 and 2 C 2) specific for antigenic site I recognize the same epitope, i.e., the genus specific one. These MAbs have broad reactivity patterns, because they reacted with the hexons of all human AV types studied by ELISA and HA (2), and with bovine AV type 3 in gel precipitation experiment (3). Their specificity to sterically independent epitopes comparing to MAbs H 12, 2 A 1, and 1 B 2 were also demonstrated by gel diffusion experiments. MAb H 12, however reacted with all human AV types tested, failed to react with bovine AV type 3 both in ELISA and HA (unpublished data), as well as in gel diffusion experiments (3). This MAb did not inhibit the binding of the two MAbs specific for antigenic site I and showed only unidirectional complete blocking of the MAbs specific for the antigenic site II. These findings suggest that the epitope recognized by MAb H 12 should be considered as a sterically distinct antigenic determinant (site III).

On the other hand, competition-binding ELISA showed that a large number of cross-reacting epitopes are present on the adenovirus hexon. At least seven epitope clusters on the antigenic site II determined by fifteen MAbs show some similarity to each other on the basis of different kinds of complete or mutual unidirectional inhibition patterns. The cause of the nonreciprocal (unidirectional or one-way) inhibition could be a steric change of the epitope due to the attached MAb, which than prevents partially the binding of the labelled MAbs. Neighbouring epitopes may be located in a groove on the surface of the antigenic site, therefore the binding of the competing MAb could sterically block the access of the labelled MAb specific for a different

epitope in the groove (7, 8, 10, 15). A further explanation could be the differences due to antibody avidities (16).

The analysis of the antigenic structure of the adenovirus hexon leads to two major conclusions. On the one hand, the nine epitopes (epitope clusters) identified by eighteen MAbs can be located on three distinct presumably nonoverlapping antigenic sites. On the other hand, the data suggest that seven epitope clusters identified by fifteen MAbs on the antigenic site II contain partially overlapping epitopes and may be a part of a large single immunodominant region on the AV 1 hexon as well as on the heterologous hexon types, as suggested by the experiments with heterologous hexons.

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