Serologic Demonstration of the Albuminoid Nature of the B700 Murine Melanoma Antigen (43261)

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Abstract. **Limited available evidence indicates that the B700 murine melanoma antigen is related to serum albumin, but potential relationships to other members of the serum albumin protein family have not yet been established. Using specific antibodies raised against each of the members of the albumin family, we have studied cross-reactivity by solid phase enzyme-linked immunosorbent assay and Western immunoblotting. We demonstrate that B700 is serologically cross-reactive to members of the serum albumin family, which includes α-fetoprotein and vitamin D binding protein. Therefore, B700 is part of the serum albumin family of proteins, although the mechanism underlying its specific expression by transformed melanocytes remains unknown.**

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For almost 40 years now, it has been understood that the immune rejection of tumors in experimental animals is mediated by antigens unique to the tumor cells, rather than by histocompatibility antigens (1). However, surprisingly few "complete tumor antigens" have been described to date. In this context, a "complete" antigen is one which is (i) tumor specific, (ii) present on all individual tumors of the same histotype, (iii) capable of eliciting a tumor rejection response, and (iv) an antenna molecule recognized by the tumor-bearing host. Of the complete antigens identified to date, even fewer are known to be related to normally occurring proteins. In this regard, melanoma tumors are of particular interest because four types of tumor proteins related to normal proteins have been identified (2–5, and reviewed in 6).

The B700 murine melanoma antigen is especially intriguing because it is both a complete tumor antigen and is related to two normal proteins. Fingerprint mapping of CNBr fragments demonstrated that B700 is a multiple deletion variant of C700, an 81-kDa membrane protein found in normal cutaneous melanocytes (7). Subsequently, B700 was found to be related to, but distinct from, serum albumin (5). That B700 is recognized as a tumor antigen has been shown by the fact that melanoma-bearing mice produce antibodies to B700 (8) and that immunization with B700 can elicit specific tumor rejection in the immunized mice (9).

Three limited types of evidence, structural, functional, and immunochemical, suggest the relationship of B700 to serum albumin. The structural evidence is that B700 is related to serum albumin by amino acid composition—the S delta Q method of compositional analysis indicates that the two molecules are related yet distinct. Moreover, there is 40% identity in the first 15 residues of N-terminal amino acid sequence (5); complete sequence data are not available at this time. Functional studies have demonstrated that the ability of murine serum albumin to catalyze the interconversion of prostaglandins has been conserved in B700 (10). Immunochemically, mice immunized to melanoma tumors produce antibodies which recognize B700 and also cross-react with murine serum albumin (8). Rat monoclonal antibodies to B700 cross-react with serum albumin as well (11).

Although the above observations point to the similarities between B700 and serum albumin, the relationship between B700 and the other members of the serum albumin protein family, i.e., α-fetoprotein (AFP) and vitamin D binding protein (DBP), has yet to be established. It is important to study these relationships be-

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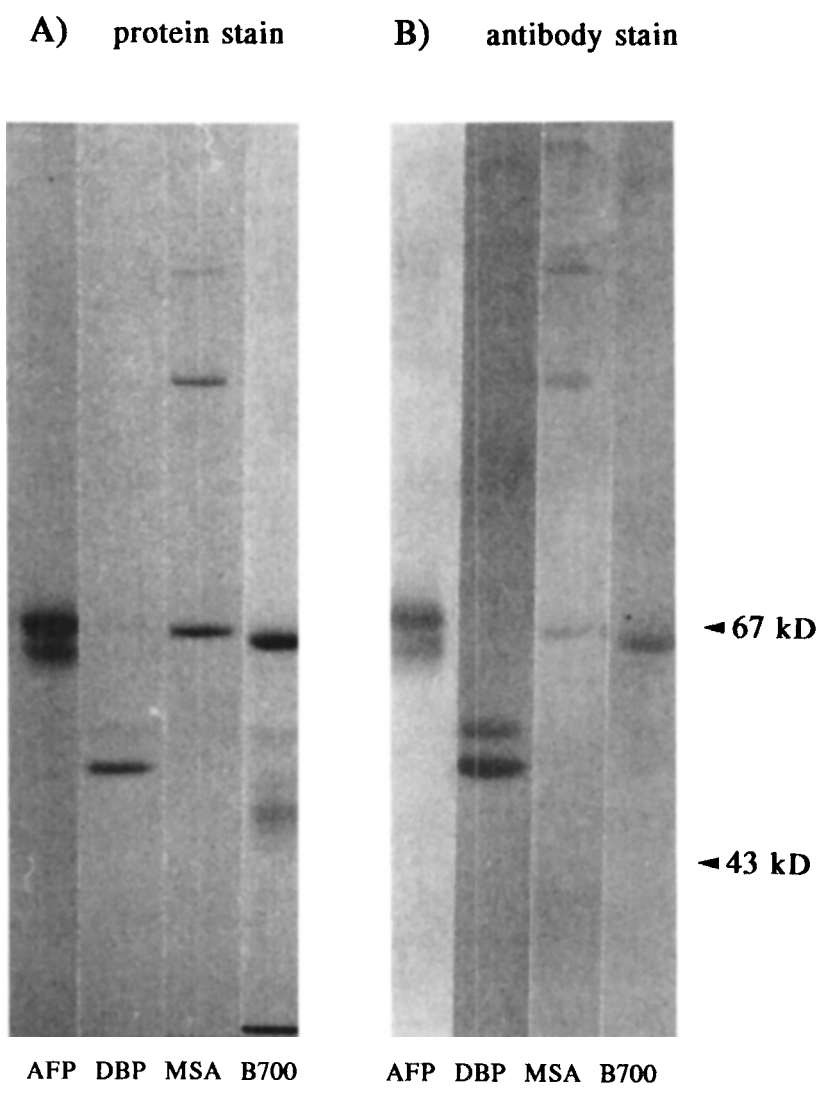

Figure 1. Purity of antigens and specificities of antibodies used. Antigens as noted at the bottom were separated by sodium dodecyl sulfate-gel electrophoresis (3 μg/lane), and stained for protein content (A) or transferred to nitrocellulose and reacted with the specific antisera (B), as detailed in Materials and Methods.

cause they may yield valuable clues as to the mechanisms tumor cells use to produce antigenic proteins. Consequently, we have studied the serologic cross-reactivity of B700 to murine serum albumin (MSA), DBP, and AFP. It will be shown by immunologic criteria that B700 is, indeed, a bona fide member of the serum albumin protein family.

Materials and Methods

Target Proteins and Antibodies. B700 protein was purified from homogenized B16F10 melanoma tumors by preparative gel electrophoresis, under non-reducing conditions, as described previously (8, 9). Fraction V MSA was purchased from Sigma Chemical Co. (St. Louis, MO). Murine AFP was obtained from Calbiochem Corp. (La Jolla, CA). Murine DBP, affinity-purified on an actin column, and a specific rabbit antiserum to murine DBP (α-DBP), were kind gifts from Dr. J. Haddad, University of Pennsylvania, Philadelphia, PA (12, 13). Bovine hemoglobin (Hb), used routinely as a background control, was a gift from Dr. D. Kasbekar, Georgetown University, Washington, DC. The purity of the proteins were verified by sodium dodecyl sulfate-gel electrophoresis according to Laemmli (14). Rabbit antibodies to B700 (α-B700) were from the same preparation used in our previous studies, where their specificities and titers are reported in detail (15–17). Rabbit antisera, and affinity-purified IgG, to MSA (α-MSA) and to AFP (α-AFP) were gifts from Dr. B. Ledford, Medical University of South Carolina, Charleston, SC (18). Normal rabbit serum and peroxidase-conjugated antirabbit IgG were obtained commercially from DAKO Corp. (Carpinteria, CA).

Enzyme-Linked Immunosorbent Assay. The enzyme-linked immunosorbent assay was performed as previously detailed (11). Briefly, 100 ng of target pro-

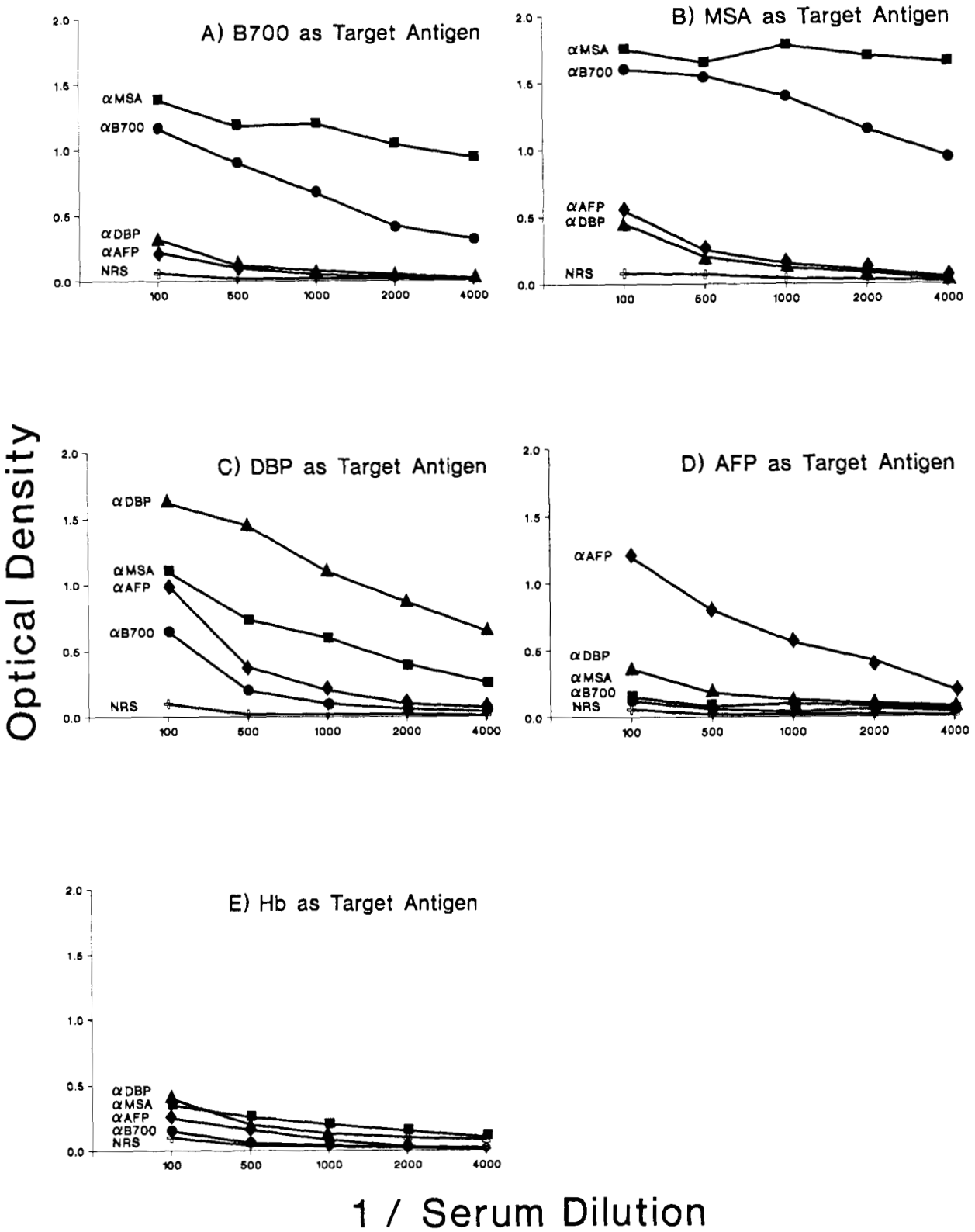

Figure 2. Antibody reactivity against immobilized target antigens. Antigens as noted were bound to microtiter plates and then reacted with antisera (at the dilutions noted) as detailed in Materials and Methods. Target antigens bound to the plate were A, B700; B, MSA; C, DBP; D, AFP; and E, Hb.

Table I. Amino Terminus Sequence Homology in the Serum Albumin Family[a]

Protein	Amino terminus-residue number														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
B700 #1	G	T	F	I	L	E	I	A	H	S	F	K	D	R	G
B700 #2	D	T	H	K	S	E	I	A	H	R	F	K	D	L	G
B700 #3	E	A	—	K	S	E	I	A	A	R	Y	N	P	L	—
MSA	E	A	H	K	S	E	I	A	H	R	Y	N	D	L	G
AFP	A	L	H	E	N	E	F	G	I	A	S	T	L	D	S
DBP	L	E	R	G	R	D	Y	E	K	D	K	V	C	Q	E

[a] Primary sequence data for B700 and MSA were taken from (5); data for AFP and DBP were taken from (23).

tein/well were bound to the wells of 96-well Immulon II plates in carbonate binding buffer. Following evaporation to dryness, the carbonate residue was dissolved, and excess binding sites in the wells were blocked with 100 μg of soluble fish gelatin (Norland Products, New Brunswick, NJ)/well overnight at 4°C. After incubation for 1 hr at 37°C with the appropriate antisera, diluted in phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween) as noted in the tables and figure legends, the wells were washed thoroughly with PBS-Tween and then incubated with peroxidase-conjugated second antibody, diluted at 1/1000 in PBS-Tween, for 30 min at 37°C. Binding was quantified by reaction with *o*-phenylenediamine substrate in the presence of H_2O_2 and detection in a Bio-Rad model 2550 EIA Reader at 492 μm, using PBS controls as blanks. Bovine hemoglobin was used as a negative target control and normal rabbit serum was used as a negative antibody control; for each experiment, serial dilutions of antisera from 1/100 to 1/4000 were evaluated. For immunocompetition experiments, 10 μl of rabbit antiserum were preincubated for 1 hr at 37°C with 10 μl of purified B700 (5 mg/ml) or with PBS-Tween. After the preincubation, the sera were diluted as noted in Table II and assayed as described above. Assays were always performed at least in duplicate; each datum point reported in the figures and tables represents the mean of four determinations, and standard errors of the means were always less than ±10% (in most instances smaller than the symbols used in the figures). Statistical significance was assessed using Student's *t* test, and differences were considered significant if $P < 0.05$.

Western Immunoblotting Protocols. Three micrograms per lane of each antigen were separated by sodium dodecyl sulfate-gel electrophoresis and transferred to nitrocellulose membranes, as detailed previously (11). Proteins were visualized with Coomassie blue staining. Specific reactivity of the antibodies with antigens was determined, after blocking of additional binding sites with gelatin, by binding of the primary antibody (at 1/200 dilution) and subsequent visualization by reaction with a peroxidase conjugated secondary antibody, as noted previously (11).

Results and Discussion

The purity of the antigens used in this study, and the specificity of the antibodies raised against those antigens, can be seen in Figure 1. Under the nondenaturing conditions employed for the electrophoresis, it can be seen that MSA polymerizes and that its antibodies recognize those polymers, whereas B700 does not polymerize. In order to establish whether B700 is related to the other proteins of the serum albumin family, we have performed symmetrical experiments in which the antibodies raised against B700, MSA, DBP, or AFP were tested for cross-reactivity to those various antigens. For the experiment shown in Figure 2A, the five sera were evaluated for binding to B700-coated wells. The results indicate appreciable binding for both the α-B700 and α-MSA antisera, with α-MSA being the highest titer. Antibodies to AFP or DBP did not bind to B700 above background level (compare Fig. 2E below). Likewise, in experiments using MSA bound to the solid phase (Fig. 2B), antibodies to MSA or B700 bound in higher amounts to MSA than did antibodies prepared against AFP or DBP, although some binding was observed with α-AFP above background. The two noteworthy observations from these experiments were (i) α-MSA binding was higher than α-B700 to MSA or B700 antigen, indicating that α-MSA is the stronger antiserum, and (ii) the similarity of the binding patterns of the various antisera to both B700 and MSA as target antigens suggests that B700 is as closely related to AFP and DBP as is MSA.

When DBP was bound to the solid phase, the highest titer of binding was by the antibodies raised against murine DBP (Fig. 2C), although antibodies raised against MSA or AFP also reacted with the DBP, above background, as did B700 antibodies at the highest titer. It is not clear why cross-reactivity between B700 and DBP is so weak, considering that the cross-reactivity of the other members are relatively strong. Antibodies to B700 reacted with DBP to a greater extent than antibodies to DBP reacted with bound B700 (shown above in Fig. 2A). The order of binding to DBP was α-DBP > α-MSA > α-AFP > α-B700. Finally, when AFP

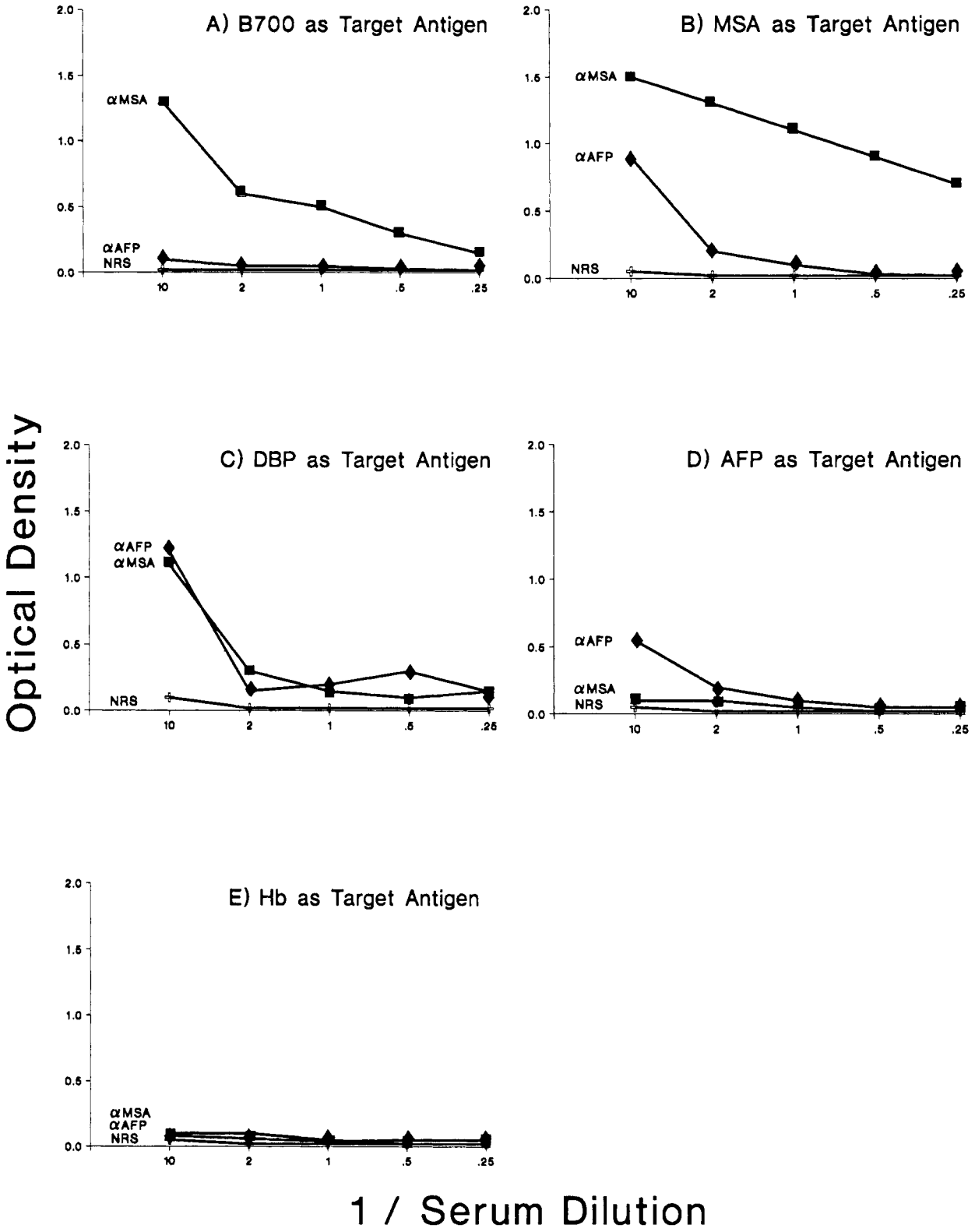

Figure 3. Reactivity of affinity-purified antibodies against the target proteins. Affinity-purified antibodies as noted were used in enzyme-linked immunosorbent assays for reactivities of antigens bound to the plates exactly as detailed for Figure 1.

Table II. Immunocompetition of Antibody Binding

Antigen bound[a]	Normal rabbit serum		α-B700 + PBS		α-B700 + B700		% Inhibition[b]	
	1/250	1/1000	1/250	1/1000	1/250	1/1000	1/250	1/1000
Experiment 1								
Hb	0.09	0.03	0.10	0.05	0.13	0.06	—	—
B700	0.17	0.04	0.28	0.15	0.26	0.08	18	64
MSA	0.12	0.07	0.66	0.37	0.19	0.09	87	94
DBP	0.09	0.02	0.11	0.06	0.13	0.04	—	50
AFP	0.10	0.04	0.08	0.04	0.11	0.06	—	—
Experiment 2								
Hb	0.05	0.01	0.09	0.04	0.11	0.04	—	—
B700	0.05	0.02	0.16	0.07	0.07	0.03	82	80
MSA	0.04	0.02	0.46	0.32	0.15	0.06	74	87
DBP	0.04	0.01	0.07	0.03	0.08	0.03	—	—
AFP	0.01	0.00	0.01	0.00	0.04	0.03	—	—

[a] Antigens as noted were bound to enzyme-linked immunosorbent assay plates and assayed for competition of antibody binding (at the dilutions indicated) after preincubation with or without B700 antigen, as detailed in Materials and Methods.
[b] Percentage of competitive inhibition of antibody binding in the presence of B700 antigen, following subtraction of nonspecific binding by normal rabbit serum.

was bound to the solid phase, the only significant binding was displayed by antibodies raised against AFP (Fig. 2D); levels of binding to AFP by the α-DBP, α-MSA and α-B700 antibodies were not significantly above background. The negativity of the α-MSA serum is a surprising result, in view of the known sequence homology and reciprocal developmental relationship of AFP to MSA (Table I) (19–21). It is especially surprising given that the α-MSA is a strong antiserum. Binding of these same five antisera to purified Hb (Fig. 2E), used as a control, was uniformly low, or negative, as expected. The suitability of Hb to serve as a negative control was verified by experiments in which soluble Hb was ineffective as a potential competitor during the antibody incubation (not shown). These experiments also serve as a specificity control, indicating that the antibodies do not bind irrelevant proteins in solution.

In consideration of the negative results obtained for binding of the α-MSA serum to AFP (Fig. 2D), we obtained affinity-purified rabbit antibodies to MSA and AFP and tested them against the different targets (Fig. 3). These results, in which purified IgG are compared on an equal protein basis, agree with the data presented above (in Fig. 2), i.e., that α-MSA antibodies cross-react well with B700, MSA, and DBP, but not with AFP, and that α-AFP antibodies react with MSA, DBP, and AFP, but not with B700.

To further define the nature of the cross-reactivity, i.e., whether the reactivity was against contaminants (either antigen or antibody in nature), we performed two different types of experiments, examining the specificity of the antibody recognition by competition protocols and by Western immunoblotting. In two replicate competition experiments (Table II), preincubation of antisera with purified B700 effectively competed with α-B700 binding to B700 and MSA, but not with AFP; there was some slight competition with the minor binding of that antisera to DBP (Experiment 1), although it was not statistically significant. These results paralleled exactly the enzyme-linked immunosorbent assay results presented in Figure 2.

Taken together, the experiments described above clearly demonstrate that B700 is related, by immunochemical criteria, to members of the albumin family, which, combined with data from the structural and functional studies (5, 10), indicate that B700 should be considered part of the serum albumin protein family. This is important in light of the recent postulation that the serum albumin family of proteins, by cooperative interaction of the different members, has the potential to serve as a system of cellular recognition-interaction-adhesion molecules (22). It is interesting to speculate that B700 may retain the domain structure of other albuminoids, but assessment of the precise relationships of B700 to the other albuminoids awaits complete sequence determination. The mechanism(s) underlying the specific expression of B700 by melanoma cells, whether by gene duplication, aberrant missplicing of mRNA or other related processes is currently under investigation. Such studies should yield important insights into the mechanisms tumor cells use to produce antigens that are related to normally occurring proteins.

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