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DEVELOPMENT OF MONOCLONAL ANTIBODIES TO 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE

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SUMMARY: This report describes the development of a series of monoclonal antibodies to rat liver 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR). Sera from hybridoma tumor-bearing mice were used to remove and characterize HMGR activity from a mixture of rat liver proteins. Two IgG_2 monoclonal antibodies removed separately greater than 80% HMGR activity while non-immune mouse or negative hybridoma-derived sera were ineffective. Radiolabeled immunoprecipitates of enzyme preparations resolved in one- and two-dimensional SDS-PAGE showed two predominant subunits at M_r 52,000 and 54,000. Our results indicate that in these preparations of rat liver proteins HMGR exists as a heteropolymer with at least two distinct subunits of different molecular weights.

Extensive evidence supports the conclusion that 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR) is the major regulatory enzyme in the biosynthesis of cholesterol (1). The activity of the enzyme is modulated by effectors such as dietary cholesterol, mevalonate, fasting, etc., (1-8). Current information suggests that cellular HMGR activity is altered by a change in its content and catalytic efficiency (9-15). These conclusions were derived from immunotitration analyses using polyvalent rabbit anti-rat HMGR antisera of microsomal and purified HMGR from animals and cells maintained in different physiological conditions. These analyses have also indicated that HMGR may exist in different interconvertible and irreversibly altered forms (12-15). Given the probability that the enzyme may exist in the cell as subspecies, we undertook the development of monoclonal antibodies to

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Abbreviations used: HMG-CoA, 3-Hydroxy-3-methylglutaryl-Coenzyme A; HMGR, HMG-CoA reductase; HSE, heat soluble enzyme; PBS, phosphate buffered saline; BSA, bovine serum albumin; RIA, PBS containing 1% BSA; PAGE, polyacrylamide gel electrophoresis; SAC, Staphylococcus aureus membranes; NMS, normal mouse serum.

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HMGR with the prospect of raising specific antibodies to one or more of its forms. Our development of a series of monoclonal antibodies to rat liver HMGR is described in this report. Of these, two mouse IgG_2 antibodies designated as 4D7-E10 and 3C10-F11 have been characterized further for their ability to bind and remove HMGR from a crude mixture of rat liver proteins. Our initial electrophoresis results of HMGR precipitated by the monoclonal antibodies suggest that in these preparations the enzyme exists in different molecular forms.

MATERIALS AND METHODS

Preparation of the antigen and the immunization protocol: Mice were immunized intraperitoneally with partially purified preparations of HMGR complexed with alum (16). This enzyme preparation represents material from microsomes solubilized in the absence of protease inhibitors (17,18) and processed to the heat step essentially as described by Beg et al (8) except that exposure to 65° was for only 5 min. The preparation here is referred to as heat soluble enzyme (HSE) and had a specific activity of 300 nmol MVA produced/min/mg of protein. Based on the reported specific activity of purified, homogeneous preparations of HMGR (11), the antigen (HSE) represented a 5% pure solution of HMGR. BALB/c mice were immunized three times at two week intervals, and three days after the third immunization were sacrificed to obtain the spleens.

Production of hybridomas: Spleen cells from mice injected with HSE-alum complex were hybridized with mouse NS-1 myeloma cells using 38% polyethylene glycol as the fusion agent (19,20). After three weeks of growth in HAT medium, hybridomas were screened for secretion of anti-HSE immunoglobulins.

Solid phase radioimmunoassay for selection of anti-HSE monoclonal antibodies: The assay was developed by using HSE and polyvalent rabbit anti-rat HMGR antibody (21) as described in Fig. 1.

Production of monoclonal anti-HMGR antibody containing sera: Anti-HSE antibody-secreting hybridomas were cloned and tested against purified HMGR (gift kindly provided by Dr. Z. Beg (8)) using the solid phase radioimmunoassay. A selected number of positive clones were injected subcutaneously into mice to develop tumors and obtain higher concentrations of antibody. Tumor-bearing mice were bled and their sera tested for anti-HMGR activity using Buffer A (21) and the HMGR assay procedure described by Shapiro et al (23).

RESULTS

The solid phase radioimmunoassay: A sensitive assay was designed to detect the presence of anti-HSE and anti-HMGR antibodies. In this assay, polyvalent rabbit anti-rat HMGR antibody activity could be detected at dilutions of more than 1000-fold. In addition, amounts of antigen calculated to be lower than 0.5 ug (50 ul per well of 10 ug/ml solution) were sufficient to produce a signal to noise ratio in this system that was 4- to 10-fold above

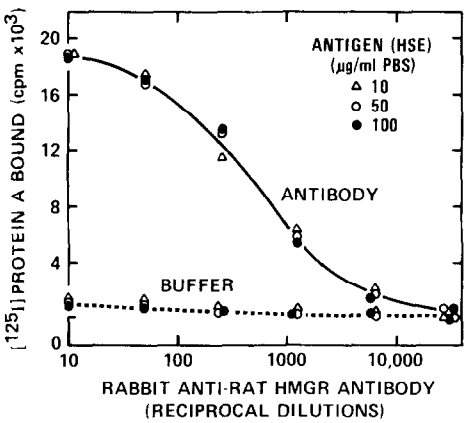


Fig. 1. Solid phase radioimmunoassay developed with polyvalent rabbit anti-rat HMGR antibody to select anti-HSE and anti-HMGR secreting hybridomas. A 96-well microtiter plate was coated with one of three concentrations of HSE, 10, 50, or 100 ug/ml, in 50 mM phosphate buffered saline (PBS) pH 8.1 that contained 50 ug/ml BSA and kept at room temperature for 1 h. Wells were washed with RIA buffer (PBS + 1% BSA) and serial dilutions of rabbit antibody in RIA buffer added. Control wells received only RIA buffer. After another hour, the wells were washed with RIA buffer and 50 ul of [^{125}I] Protein-A (22) containing 20,000 cpm added per well. An hour later, wells were washed, dried, cut off from the plate and counted in a gamma-radiation counter. To test the production of anti-HSE monoclonal antibodies, culture supernatant fluid from hybridomas replaced the polyvalent rabbit antibody.

background (Fig. 1). The assay was therefore well suited to test the presence of anti-HSE and anti-HMGR antibodies secreted by the hybridomas.

From a cell fusion in which 480 wells were plated with cells, colony growth was observed in a total of 240. Of these, 60 colonies produced anti-HSE immunoglobulins; 21 reacted with purified HMGR as indicated by counts in the assay which were three- to twelve-fold greater than background and only 8 of the 21 remained stable subsequent to cloning. Four of these stable clones produced IgG_1 class of antibodies as determined by their reactivity with monoclonal anti-IgG_1 (gift of Dr. V.T. Oi, Department of Structural Biology, Stanford) while five were positive to Protein A (Table 1).

Removal of HMGR activity by immune sera of tumor-bearing mice: Clones 7C9-F8, 3C10-F11, 4D7-E10 and 8E4-A1 were used to generate subcutaneous tumors in mice, and sera from tumor-bearing mice were tested for specific binding to HMGR. Fig. 2 compares the effectiveness of polyvalent rabbit antibody with monoclonal antibodies in removing HMGR activity from HSE preparations of rat liver. Our results showed that monoclonal antibodies 4D7-E10 as well as 8E4-

TABLE I

Hybridomas secreting anti-HSE and anti-HMGR antibodies. The immunoglobulin class of secreted proteins is described as well as their affinity for binding to Staphylococcus aureus membranes (SAC) or Protein A.

Number of primary wells			Stable clones	Class	Code	Binding to SAC or protein A
Cell Growth	Anti-HSE	Anti-HMGR				
117	29	14	4	IgG_1	8.E4	+
				"	6.C3	-
				"	3.G9	-
				"	1.C2	-
123	31	7	4	IgG_2* ...	7.C9	+
				"	4.D7	+
				"	1.D8	+
				"	3.C10	+

*IgG_{2a} IgG_{2b} IgG_3 - all classes are protein A positive.

A1 were most efficient in binding HMGR while 3C10-F11 and 7C9-F8 were of intermediate strength. Polyvalent rabbit antibody effectively removed 90% of the HSE's HMGR activity.

Subsequent studies were done only with 3C10-F11 and 4D7-E10 and immunotitrations with these antibodies demonstrated that 85-90% of HMGR activity was complexed and removed by the immunoglobulins (Fig. 3) while neither non-immune mouse nor a negative hybridoma-derived serum was effective. A 10 ul aliquot of 4D7-E10 removed 76% of the HMGR activity at 4° in 30 min.

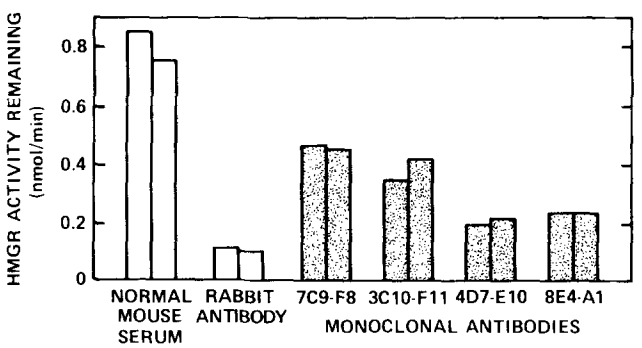


Fig. 2. Removal of HMGR activity by normal mouse serum (NMS), rabbit HMGR antibody and hybridoma-secreted immunoglobulins. Antibody was added to an HSE preparation that had been precleared with SAC adsorbent (25) and the mixture kept at 4° for 30 min. SAC pellet derived from 200 ul of a 10% w/v suspension was added to the mixture to bind the antibody-enzyme complex and after 30 min at 4° the mixture was centrifuged, and 80 ul of the supernatant removed to assay residual HMGR activity.

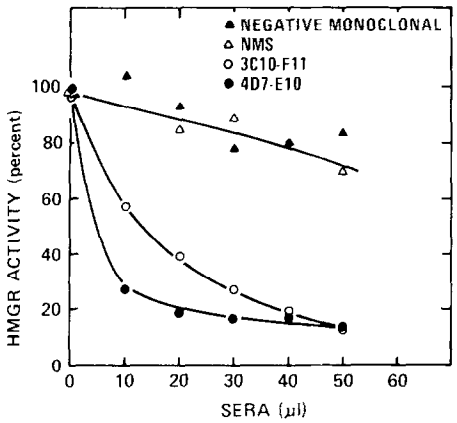


Fig. 3. Experiment was conducted essentially as described in Fig. 2, except a range of serum concentrations were used. Final volumes were adjusted with non-immune mouse sera. One hundred percent enzyme activity corresponds to a value of 1.36 nmol MVA produced/min/mg protein.

Analyses of [^{125}I]HSE proteins immunoprecipitated by 4D7-E10 and 3C10-F11 using SDS-PAGE: Fig. 4a shows a representative Coomassie Blue stained gel of an immunoprecipitate to expose the immunoprecipitated proteins. The stained polypeptides were primarily the heavy (H) and light (L) chains of the mouse and hybridoma secreted immunoglobulins in the antigen-antibody-SAC complex. Heavy chains of IgG are around M_r 55,000, and in a range similar to that reported for HMGR monomer (8,12,27,28). The presence, therefore, of any HMGR protein subunits is likely to be masked by the abundant IgG heavy chains, except that since the antigen is radioiodinated in this experiment, it was detected by autoradiography of the gels. The autoradiogram (Fig. 4b) revealed predominantly two radiolabeled polypeptides as a protein doublet of around M_r 52,000 and 54,000 immunoprecipitated by hybridomas 4D7-E10 (indicated by the arrow in Fig. 4b, lanes 1, 2, and 4) and 3C10-F11 (lane 3). Non-immune mouse serum used to immunoprecipitate showed no radiolabeled proteins (Fig. 4b, lane 5). The reported subunit size of purified HMGR has been estimated to be around M_r 50,000-53,000 (8,12,27,28), while polyvalent rabbit HMGR antibodies, raised against purified rat liver enzyme were recently shown to immunoprecipitate from Chinese Hamster Ovary cells, polypeptides with molecular weights of M_r 53,000 (29) as well as M_r 60,000 and M_r 127,000 subunits. While other faint radioactive bands are seen in these autoradiographs (Fig. 4b), presently

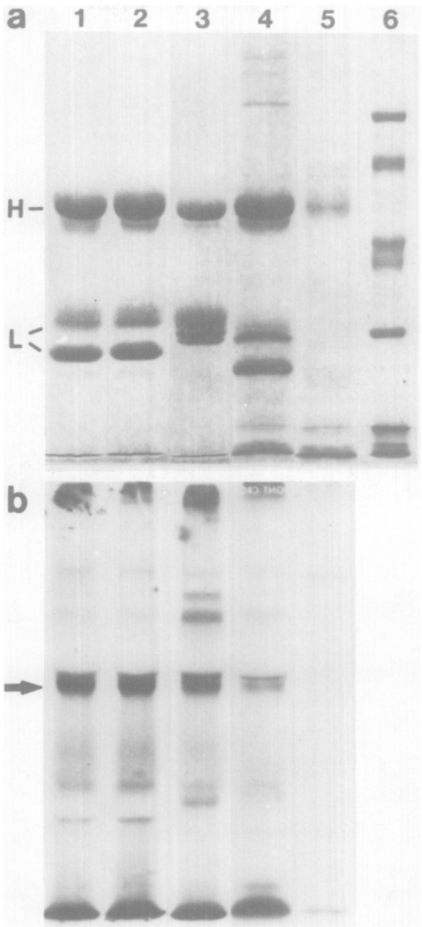
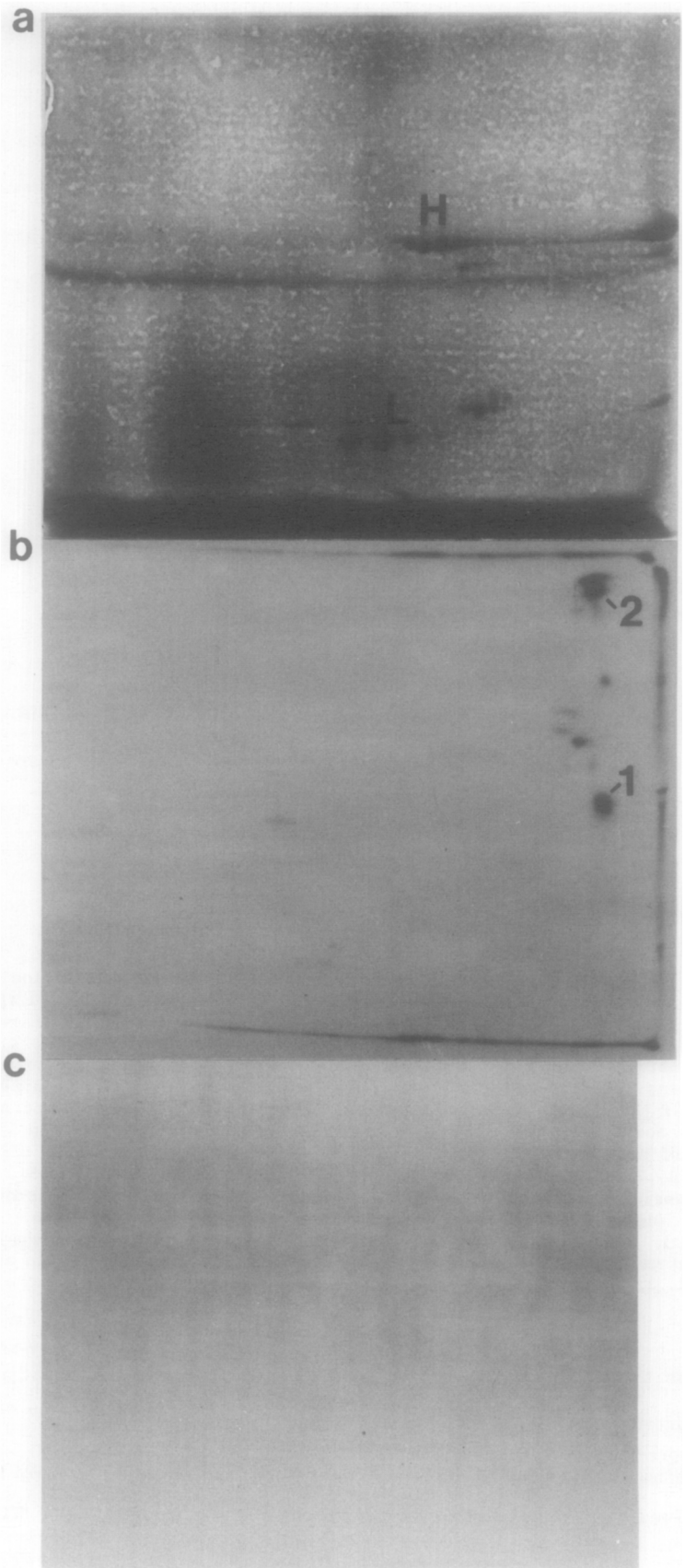


Fig. 4. Analyses of immunoprecipitated [^{125}I]HSE proteins. Radioiodinations of HSE were done using 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Iodogen, Pierce Chemicals). Preparations precleared with SAC were incubated with selected antisera for 30 min at 4^{o} and complexed to SAC (24). The [^{125}I]antigen-antibody-SAC complex was dissociated with SDS-2,mercaptoethanol-containing buffer, heated at 100^{o} for 2 min and resolved in one-dimensional SDS-PAGE (26). Fig. 4a is the Coomassie Blue stained protein pattern of the SDS-10% acrylamide gel showing relative mobilities of heavy (H) and light (L) chains of hybridoma and mouse immunoglobulins with 10, 20, and 5 ul of 4D7-E10 (lanes 1, 2, 4), 30 ul of 3C10-F11 (lane 3) and 20 ul of NMS (lane 5). Lane 6 has a series of molecular weight standards (BioRad): 98,000 (phosphorylase); 68,000 (BSA); 48,000 (ovalbumin); 30,000 (carbonic anhydrase); 20,000 (trypsin); and 15,000 (lysozyme). Fig. 4b is the autoradiogram of the protein stained gel. The two prominent radiolabeled bands marked by the arrow in lanes 1, 2, 3 and 4 are at M_r 52,000 and 54,000.

their identities are not known. Whether they represent contaminating proteins or are related to HMGR is to be determined.

In addition to the one-dimensional PAGE analyses, immunoprecipitates were also resolved by the two-dimensional procedure of Jones (25). With protein stain alone (Fig. 5a), the IgG heavy (H) and light (L) chains were observed.


a
H
L
b
2
1
c

Autoradiographs of this gel (Fig. 5b) showed that radioactivity was not present in the IgG heavy or light chains but was mostly associated with protein doublets, one of around M_r 52,000-54,000 (Fig. 5b,1) and another >100,000 (Fig. 5b,2). Non-immune mouse serum at the same concentration did not complex any radiolabeled proteins (Fig. 5c). The radiolabeled proteins were distinctly separated from the immunoglobulin chains in these gels and verified that radioactivity in the 52,000-54,000 doublet of Fig. 4b was not a consequence of a co-migration of non-specific radioactivity with immunoglobulin heavy chains. In general, the radiolabeled protein pattern of the two-dimensional PAGE was compatible with that observed in the one-dimensional gels.

DISCUSSION

This is the first report describing the production of monoclonal antibodies to HMGR. The antigen used to elicit antibody production was a mixture of proteins of which HMGR was one component. The prime consideration in using this antigen preparation was the assumption that HMGR may exist as several subspecies (12-15), and if so, the probability of raising antibodies to different forms was greater with a heterogeneous than a homogeneous preparation. As demonstrated in Fig. 1, we have isolated several hybridomas that secrete anti-HMGR antibodies. Of these, two monoclonals, 4D7-E10 and 3C10-F11, have been described here and these effectively bind and remove HMGR activity from a solubilized mixture of rat microsomal proteins. Radioiodinated HSE preparations immunoprecipitated and separated by one- and two-dimensional polyacrylamide gel electrophoresis revealed that HMGR consists of subunits with M_r predominantly at 52,000 and 54,000. These

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Fig. 5. Resolution of [^{125}I]HSE proteins immunoprecipitated with 4D7-E10 and NMS on two-dimensional PAGE. Radioiodinated antigen-antibody complexes were adsorbed to SAC, extracted with isoelectric focusing-sample buffer (25) and first resolved in a non-equilibrium pH gradient of 4.5 to 9 with 4 percent acrylamide. The proteins were then separated by size in the second dimension in SDS-10% acrylamide. Fig. 5a is a Coomassie Blue stained gel of immunoprecipitated proteins with 10 ul of 4D7-E10 serum. Heavy (H) and light (L) chains of immunoglobulins are seen. Gels were exposed to x-ray film for two days and Fig. 5b is the autoradiogram of radioiodinated proteins complexed to 4D7-E10 and Fig. 5c to NMS. The 52,000-54,000 protein doublet (1) and the >100,000 polymer (2) isolated with 4D7-E10 are marked. NMS at the same concentration appeared not to complex any proteins (Fig. 5c).

molecular weight estimates of the enzyme are comparable with the reported molecular size of the monomers purified in the absence of protease inhibitors (8,12,18,27,28). The nature of the higher molecular weight bands in Fig. 4b and spots in Fig. 5b is not clear. Others (29) have reported polypeptides at M_r 127,000 and 60,000 in immune precipitates of [^{35}S] methionine-labeled extracts of Chinese Hamster Ovary cells with polyvalent rabbit antibody to homogeneous HMGR. It is clear that if protease action is a cause for the production of several different sized molecular species, then the action of these degrading enzymes will have to be controlled during HMGR preparations before an assessment of the number of HMGR species in vivo can be made.

Further investigations are underway to explore the ability of the antibodies to recognize enzymes isolated in protease-inhibited preparations (17,18) from different metabolic states. In addition, these antibodies will be used to study the differential turnover of HMGR in normal and experimentally treated cells challenged with modulators such as oxygenated sterols, fetal calf serum, compactin (4-6) or mevalonate (2,14).

REFERENCES

1. Rodwell, V.W., Nordstrom, J.L., and Mitschelen, J.J. (1976) Adv. Lipid Res. 14, 1-74.
2. Edwards, P.A., Popjak, G., Fogelman, A.M., and Edmond, J. (1977) J. Biol. Chem. 252, 1057-1063.
3. Gould, R.G. (1977) in "Cholesterol Metabolism and Lipolytic Enzymes" (Polonovski, J. ed.) pp. 13-38, Masson Incorporated, France.
4. Kaneko, I., Shimada, H.Y., and Endo, A. (1978) Eur. J. Biochem. 87, 313.
5. Brown, M.S., Faust, J.R., Goldstein, J.L., Kaneko, I., and Endo, A. (1978) J. Biol. Chem. 253, 1121.
6. Alberts, A.W., Chen, J., Kuron, G., Hunt, E., Juff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Schonberg-Albers, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Leisch, J., and Springer, J. (1980) Proc. Natl. Acad. Sci. USA 77, 3957.
7. Ingebritsen, T.S., Geelen, M.J.H., Parker, R.A., Evensen, K.J., and Gibson, D.M. (1979) J. Biol. Chem. 254, 9986-9989.
8. Beg, Z.H., Stonik, J.A., and Brewer, H.B. (1980) J. Biol. Chem. 255, 8541-8545.
9. Beirne, O.R., Heller, R.A., and Watson, J.A. (1977) J. Biol. Chem. 252, 950-954.
10. Hardgrave, J.E., Heller, R.A., Herrera, M.G., and Scallen, T.J. (1979) Proc. Natl. Acad. Sci. USA 76, 3834.
11. Edwards, P.A., Lemongello, D., and Fogelman, A.M. (1979) Biochem. Biophys. Acta 574, 123-135.
12. Edwards. P.A., Lemongello, D., Kane, J., Schechter, I., and Fogelman, A.M. (1980) J. Biol. Chem. 255, 3715-3725.

13. Kleinsek, D.A., Jabalquinto, A.M., and Porter, J.W. (1980) J. Biol. Chem. 256, 3918-3923.
14. Arebalo, R.E., Hardgrave, J.E., Noland, B.J., and Scallen, T.J. (1980) Proc. Natl. Acad. Sci. USA 77, 6429-6433.
15. Arebalo, R.E., Hardgrave, J.E., and Scallen, T.J. (1981) J. Biol. Chem. 256, 571-574.
16. Mishell, R.I. (1980) in "Selected Methods in Cellular Immunology" (Mishell, B.B., and Shiigi, S.M. eds.) pp. 28-68, W.H. Freeman and Co., San Francisco.
17. Ness, G.C., Way, S.C., and Wickham, P.S. (1981) Biochem. Biophys. Res. Commun. 102, 81-85.
18. Chin, D.J., Luskey, K.L., Anderson, R.G.W., Faust, J.R., Goldstein, J.L., and Brown, M.S. (1982) Proc. Natl. Acad. Sci. USA 79, 1185-1189.
19. Kohler, G., and Milstein, C. (1975) Nature 256, 495.
20. Oi, V.T., and Herzenberg, L.A. (1980) in "Selected Methods in Cellular Immunology" (Mishell, B.B., and Shiigi, S.M. eds.) pp. 351-372, W.H. Freeman and Co., San Francisco.
21. Heller, R.A., and Shrewsbury, M.A. (1976) J. Biol. Chem. 251, 3815-3822.
22. Oi, V.T., and Herzenberg, L.A. (1979) Molecular Immunol. 16, 1005-1017.
23. Shapiro, D.J., Nordstrom, H.L., Mitschelen, J., Rodwell, V.W., and Schimke, R.T. (1974) Biochem. Biophys. Acta 370, 369-377.
24. Ivarie, R.C., and Jones, P.P. (1979) Anal. Biochem. 97, 24-35.
25. Jones, P.P. (1980) in "Selected Methods in Cellular Immunology" (Mishell, B.B., and Shiigi, S.M. eds.) pp. 398-440, W.H. Freeman and Co., San Francisco.
26. Laemmli, U.K. (1970) Nature 227, 681-684.
27. Kleinsek, D.A., and Porter, J.W. (1979) J. Biol. Chem. 254, 7591-7599.
28. Ness, G.C., Spindler, C.D., and Moffler, M.H. (1979) Arch. Biochem. Biophys. 197, 493-499.
29. Sinensky, M., Torget, R., and Edwards, P.A. (1981) J. Biol. Chem. 256, 11774-11779.