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Immunology

Immunization *in vitro* and production of monoclonal antibodies specific to insoluble and weakly immunogenic proteins

(hybridoma/mitogen/antigen presentation/chromosomal protein/immune suppression)

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ABSTRACT **A procedure is described for immunizing *in vitro* and stimulating proliferation of specific B-cell lymphocytes. The method is applicable to production of monoclonal antibodies against proteins that are soluble only in denaturing solvents. An induction period is described in which antigen is presented to the B-cell population in the absence of serum. Also, antigen is coupled to mitogenic silica, which allows the effective presentation of both soluble and insoluble antigens. The results indicate hybridomas can be obtained that secrete IgMs directed against highly conserved or weakly immunogenic antigens.**

Now that the potential applications of hybridoma technology are appreciated (for review, see ref. 1), the coupling of *in vitro* stimulation of lymphocytes to hybridoma production is of great importance, as *in vitro* immunization has several advantages over *in vivo* methods. For example, (*i*) it is possible to produce antibodies directed against highly conserved or weakly immunogenic determinants (2), (*ii*) comparatively little antigen is required (3), and (*iii*) *in vitro* stimulation (4, 5) will in many cases be the procedure required to generate hybridomas secreting human antibodies. Considerable advances have been made recently in coupling the two procedures (2); however, several problems remain. The most significant is competition of introduced antigen with serum proteins or determinants in the rabbit or fetal calf serum required in most systems to support B-cell division (6). Serum-free *in vitro* stimulation systems are known (7, 8), but these rely on replacements which themselves include other proteins or immunogenic contaminants, and these systems are generally less supportive of B-cell division. Also, it has been difficult to introduce insoluble antigen into the *in vitro* stimulation reaction in a form that can be presented to the majority of lymphocytes or monocytes. Insoluble protein antigens either must be solubilized in cytotoxic solvents, which must be avoided during *in vitro* stimulation, or must be used as precipitates. Furthermore, the T-cell suppressor activity in the splenic cell population ideally should be eliminated, although it has been suggested that *in vitro* immunization is in part free of normal immune suppression (6, 9). Here we describe studies directed at these problems.

METHODS

Coupling of Antigen to Silica. Metaphase chromosomes and nuclei were isolated from HeLa cells as described (10, 11) and associated nonhistone chromosomal proteins (NHCPs) were fractionated by $NaDodSO_4$ gel electrophoresis (10–12). Single bands in stained polyacrylamide gels (13) were cut from the gel with a razor blade. The protein was eluted from the gel slice essentially as described (14) by crushing the gel and mixing for 24–48 hr with 1–5 ml of 2% $NaDodSO_4$/1% 2-mercaptoethanol/5 mM Tris·HCl, pH 7.2, and then was heated to 100°C for 2 min. The gel fragments were eliminated by centrifuging and filtering the solution through a Miller-HA filter (Millipore). Vimentin and a M_r 210,000 nucleolar protein were similarly prepared as single electrophoresis bands from HeLa cells. Total NHCPs were similarly solubilized in the above $NaDodSO_4$ solution. Fumed SiO_2 (10–1000 μg; Sigma, size 0.007 μm) suspended in water by sonication was then added to the solubilized protein and mixed for 12–24 hr at room temperature. Binding to the beads was usually rapid, but in some cases, to insure better binding, solid CCl_3COOH was added to a final concentration of 25% (the latter step is required with certain proteins in $NaDodSO_4$ solution). The silica beads with attached protein were collected by centrifugation (10,000 × *g* for 5 min) and washed repeatedly with distilled water to remove $NaDodSO_4$ and/or CCl_3COOH. More than 90% of all ^{125}I-labeled NHCP or vimentin cosedimented with the silica. Prior to use as an immunogen, the silica–antigen complex was briefly sonicated and could be sterilized by irradiation or autoclaving.

Summary of the *in Vitro* Stimulation Reaction. Spleen cells, thymocytes (isolated by screening through wire mesh), and macrophages were isolated (the latter by peritoneal cavity wash) from four or five BALB/c mice or C57/B1 × BALB/c F_1 hybrids (see refs. 16 and 17). Then, two sets of media were constructed. First, 5.0 ml of RPMI 1640 medium containing 40% fetal calf serum and all of the components described in Table 2 was thymocyte-conditioned by growing with 2×10^8 autologous thymocytes per ml. Second, 8 ml of the same medium minus the fetal calf serum and thymocytes were used to suspend the spleen cells (2.5×10^8), macrophages (1×10^7), and 500 μg of silica–antigen complex. Both cell suspensions were placed in 25-cm^2 flasks (Corning) and incubated 7–9 hr at 37°C in 5% CO_2/95% air. The thymocytes were then removed from the serum-containing medium by centrifugation, and the medium was added to the spleen cell suspension culture. Incubation continued for 5–8 days. The flask was not disturbed during incubation.

Fusion with Myeloma Cells and Selection of Hybridomas. Cells in the flask were decanted by gentle shaking. Both the myeloma (X63-Ag8.6.5.3, Ns-1, Sp2/0, or PAI-O) and spleen cell suspensions were washed twice at 37°C with RPMI 1640 medium. The number of nonreticulocyte splenic cells excluding trypan blue was determined, and they were mixed with an equal number of myeloma cells and washed one more time. The cells were fused with 37% polyethylene glycol (Merck, M_r 4000) by described procedures (18). Cells were plated at a concentration of $2–4 \times 10^5$ myelomas (determined from prefusion input) per microtiter plate well ($6–12 \times 10^5$ cells per ml) in the presence of 2×10^5 thymocytes per well in 20% fetal calf serum without HAT components (hypoxanthine/thymidine/aminopterin). At 24 hr after the

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Abbreviation: NHCP, nonhistone chromosomal protein.

fusion, the medium was replaced with HAT medium (see ref. 17). Selection for HAT resistance was complete in 5–7 days depending on the myeloma. Screening commenced at 14–20 days after fusion, as described in Table 1. Selected colonies were subcloned by limiting dilution. We have noted no striking differences in fusion efficiency with any of the four myelomas. Myelomas were routinely tested for mycoplasm contamination as this is a frequent cause of poor hybridoma efficiency.

RESULTS

Presentation of Antigen. The most appropriate means for presentation of antigen to lymphocytes *in vitro* are uncertain. The problem is exacerbated when antigens are insoluble in physiological buffers. We chose to couple protein antigens to small particulate silica, as silica has been shown to be mitogenic (19–21) and possesses a property of binding strongly to protein. Protein antigens were bound to silica in solvents in which the proteins are soluble to permit a uniform distribution. The studies described here emphasize NHCPs as antigens and, in particular, one of the major proteins of the isolated chromosomal scaffold called sc-1 (10). Bound protein was resistant to dissociation by repeated washing in physiological buffers or heating in $NaDodSO_4$/2-mercaptoethanol. The silica–protein beads were introduced into the *in vitro* stimulation reaction, where they distributed uniformly and effected the clumping of lymphocytes.

Table 1. The effect of priming in the absence of fetal calf serum on the number of antigen-specific clones

Antigen	Exp.	Induction	Clones, no.	Specific clones, %
sc-1 (2.5 μg)	1	Yes (6 hr)	93	15
	2	No	141	0
Vimentin (0.1 μg)	1	Yes (9 hr)	97	71
	2	No	117	47
Cu^{+2}-NHCP (50 μg)	1	Yes (8 hr)	61	48
	2	No	79	16

BALB/c × C57/B1 F_1 mouse spleen cells (4×10^8) were equally divided into two stimulation reactions, each containing antigen coupled to silica. One reaction mixture (Exp. 2, containing 2×10^8 cells) was incubated continuously with thymocyte-conditioned medium containing fetal calf serum and all components described in Table 2, the other reaction (Exp. 1) undergoing the specified induction period (hours specified in the absence of fetal calf serum) prior to the addition of allogenic thymocyte-conditioned fetal calf serum. After 7–9 days of growth, the lymphocytes were fused to PAI/O myeloma cells, and hybridomas were grown in 96-well microtiter dishes. Wells positive for growth were screened for antibody secretion by filtering culture supernatants through nitrocellulose paper using a filtration manifold (Schleicher & Schuell). The nitrocellulose sheet was then probed for absorbed mouse antibodies by incubating with 2×10^6 cpm of ^{125}I-labeled sheep anti-mouse IgG (Fab fragment; Amersham) as described (11). The dried nitrocellulose sheet was exposed to x-ray film to identify by autoradiography dots corresponding to antibody-producing hybridomas. Antibodies directed against specific proteins were further screened by using a similar "dot-blot" assay in which the purified protein immobilized on nitrocellulose was incubated in the manifold with hybridoma supernatants and washed, and then the entire sheet was incubated with ^{125}I-labeled sheep anti-mouse IgG, all as just described. Hybridomas screening as positive for chromosomal proteins were further screened for specific binding of antibodies to nuclei or chromosomes by an immunofluorescence assay using fixed, permeabilized HeLa cells as described (11). Specificity of all of the anti-sc-1 and some of the anti-vimentin antibodies was confirmed by using the immunoblot assay (see Fig. 1). The number of specific clones described above are those secreting antibody (which average 20–30% of total clones) binding only the specific protein (sc-1 or vimentin) in the above assays. In the case of Cu^{+2}-NHCP, specificity to nuclear antigens was determined only with the immunofluorescence assay.

The introduction of silica alone stimulated lymphocytes to divide (ref. 21, Table 2). The basis for the mitogenic activity of silica may be the indirect activation of macrophages as suggested (21), but this point remains unclear. Excessive free silica is cytotoxic. The maximum amount tolerated was 500 μg per 10^8 spleen cells, as also previously found (21).

Assembly of Reaction Components. It was anticipated that the maximal response to a specific antigen might be obtained when the antigen was presented in the absence of the milieu of serum proteins. An induction or priming period was devised during which antigen was presented to the lymphocyte population in the absence of serum, but after which thymocyte-conditioned medium containing serum was added to support growth. Lymphocytes were cultured in the absence of serum for up to 9 hr without affecting significantly the final number of responding and dividing cells, but the repertoire of resulting hybridomas was altered. Induction in the absence of fetal calf serum was required for production of significant numbers of clones specific for sc-1 protein (Table 1). In four separate attempts to produce sc-1-specific hybridomas, none were obtained when immunization was in the presence of fetal calf serum without the induction period, and many were obtained when the induction in the absence of fetal calf serum was used. However, the possibility that the induction period might be less important at high concentrations of antigen has not been investigated. Although the number of clones specific for vimentin- or copper-stabilized NHCP was increased by the use of the induction period, substantial numbers of clones specific to the antigens also were observed without the fetal calf serum-free induction period. Since sc-1 protein is a relatively weak immunogen and the other tested proteins are stronger (see below), studies to see if the apparent correlation between the value of serum-free priming and immunogenicity of the antigen is observed should be carried out.

Components of the Reaction. Table 2 summarizes the relative effects of growth-supporting factors and antisuppressor T-cell drugs. It has been shown that suppressor T cells, which are normal components of the spleen cell population, are capable of inhibiting specific antibody production *in vitro* (22). Inhibition of suppressor T-cell activity would be of obvious value, especially when attempting to generate a response to highly conserved protein. Cimetidine acts on suppressor T cells bearing the surface receptor designated H2, which are histamine binding (23, 24) and may abrogate the suppressor effect (27). 1,1-Dimethylhydrazine and hydrocortisone are used to generate a wider spectrum of antisuppressor activity, while at low concentrations leaving B-cells unaffected (28, 29). Glutathione and 2-mercaptoethanol are added as growth factors (21, 30). Nonessential amino acids, glutamine, and pyruvate are commonly used as lymphocyte supporting factors (6). In different experiments, the added nucleosides increased the yield of stimulated cells by 2- to 6-fold. The addition of thymocyte-conditioned medium after the priming period was absolutely required to support lymphocyte division. Cell concentration was important. Optimal response occurred at spleen cell concentrations of 1×10^8 to 6×10^8 cells per 25 cm^2. At lower cell concentrations, the number of stimulated cells fell quickly to 10% of maximal (data not shown).

Directing the Lymphocyte Response. Three specific antigens were studied with respect to (*i*) their ability to stimulate lymphocytes, (*ii*) their respective required concentration to elicit response, and (*iii*) the effects of the purity of the introduced antigen on specific response (Table 3). The antigens differed greatly in their ability to stimulate lymphocytes. There was, for example, an apparent difference of 10^4 in molar concentration of antigen required to obtain a reasonable probability of obtaining an anti-sc-1 clone compared with obtaining an anti-vimentin clone. When the data were normal-

Table 2. Effect of different components of the *in vitro* medium on the number of stimulated lymphocytes

Exp.	Conditions	Colonies, no.	Cells per colony, no.	Stimulated cells	
				Total no.	Relative no.
A	Fetal calf serum	3.2×10^2	3–4	1.3×10^3	1
B	Thymocyte-conditioned fetal calf serum	1.4×10^3	8	1.1×10^4	8.4
C	Exp. B + cimetidine	8.6×10^3	7	6.0×10^4	46
D	Exp. C + hydrocortisone + dimethylhydrazine	7.2×10^3	6	4.3×10^4	33
E	Exp. D + mercaptoethanol + glutathione	14.1×10^3	9	1.3×10^5	100
F	Exp. E + nucleosides + hypoxanthine	28.1×10^3	8	2.2×10^5	169
G	Exp. C + nucleosides	12.3×10^3	6	7.4×10^4	56

BALB/c × C57/B1 F_1 mouse spleen cells (3×10^6) were incubated in 0.2 ml of RPMI 1640 medium containing 100 units per ml of penicillin/streptomycin, 2 mM sodium pyruvate, 1 mM sodium glutamine, and nonessential amino acids solution at 1× (GIBCO; supplied at 100×) in the presence of 1 μg of Cu^{+2}-fixed NHCP attached to 0.1 μg of SiO_2. Other similar mixtures had additions as described above at the following concentrations: 100 μM cimetidine (Sigma); 1 μM hydrocortisone (Sigma); 1,1-dimethylhydrazine (assymetric form, Fluka) at 10 μg/ml; 20 μM 2-mercaptoethanol (Sigma); 20 μM glutathione (Sigma); 20 μM each adenosine, guanosine, uridine, and cytidine; 3 μM thymidine; and 30 μM hypoxanthine. The plates were incubated 5 days, and the number of plaque-forming colonies were determined; numbers being averaged from 12 identical wells in two separate experiments. The total number of stimulated cells was determined from the average number of cells per colony (3–4 cells per colony or greater, 30 determinations per condition) multiplied by the number of plaque-forming colonies. The numbers presented may represent an underestimate, as single or double "stimulated cells" would go unscored. An "induction" period of 8 hr was used as described in text prior to the addition of thymocyte-conditioned medium. At 5 days of incubation, the stimulated cells were easily discerned as colonies or clusters of putative daughter cells.

ized on a per hybridoma basis, vimentin was 5–10 × 10^4 times more immunogenic per mol than was sc-1. The nucleolar antigen represents an immunogenic value between the extremes of sc-1 and vimentin. There appears to be an approximate correlation between eliciting amounts of antigen and the quantities of stimulated cells and, hence, specific hybridomas.

When complex mixtures of antigens are presented to lymphocytes *in vitro*, response to certain antigens is reduced. To examine this, Cu^{2+}-fixed NHCPs were tested, as this protein population consists essentially of all chromosomal proteins (58 as detected by electrophoresis) devoid of histones and proteins unable to be crosslinked by Cu^{2+} (10). Although sc-1 protein represents one of the major protein components of Cu^{+2}-fixed NHCPs (10), the ability of sc-1 to act as an immunogen was reduced in the presence of other proteins. For example, one can compare the number of sc-1-specific hybridomas produced when purified sc-1 was used as immunogen or when an equivalent amount of sc-1 mixed with other NHCPs was used instead. Since sc-1 comprised 5–10% of the total NHCPs, it seems that the immunogenicity of sc-1 was masked by or in competition with the other proteins. Therefore, purified antigen was used when possible. Stimulating spleen cells with complex mixtures of NHCPs resulted in complex patterns of hybridoma activities, with the exception of a preponderance of antibodies directed against the nucleolus (30–35% of different activities) and diverse cytoplasmic antigens (40–45%) (data not shown).

Characterization of Producing Hybridomas and Antibodies. The *in vitro* stimulation of lymphocytes induced only a primary response; hence, >99% of all the hybridomas obtained by *in vitro* stimulation protocols secreted IgM (Table 3). We produced IgG-secreting hybridomas but at present cannot rule out the possibility that a preexisting activity in the mouse spleen cell population was immortalized. IgM-secreting hybridomas are stable; twice-cloned hybridomas have been cultured for 6 months continuously without diminution of activity. Monoclonal antibodies produced by this procedure were specific for respective immunogens by the different assays described in Table 1. For example, an immunoblot analysis of total human chromosomal proteins using a typical anti-sc-1 antibody showed a clear reaction with a protein having the electrophoretic mobility of sc-1 but no detectable reaction with any other protein (Fig. 1). Most of the antibodies recognizing other antigens had similar specificity to a single protein. The antibodies generally were applicable to many experiments requiring precise recognition of a specific protein. For example, antibodies tagged with colloidal gold or with fluorescence were used to localize the intranuclear and intrachromosomal positions of sc-1 protein via electron and fluorescence microscopy. These studies will be described elsewhere.

Antibodies directed against sc-1, the M_r 210,000 nucleolar antigen, or vimentin reacted with antigens present in all mammalian cell lines tested in the immunofluorescence assay. These cell lines included human (HeLa 53; CCL 2.2), Chinese hamster ovary cell (CHO-K1; CCL 2.2), and mouse (X63-AG8.6.5.3). For example, 18 of 19 different anti-nucleolar and anti-nuclear-chromosomal antigen-specific antibodies reacted across a spectrum of mammalian species, as did nine of nine antibodies specific for cytoplasmic antigens. When these results were compared with the activities of hybridomas produced from *in vivo* immunization with identical antigens, a notable difference was seen. On average, only 50% of anti-nuclear/nucleolar activities reacted across species lines, and anti-chromosomal activities were rarely obtained (1% of producing clones when NHCPs were used as antigen; unpublished data). Similar results after *in vivo* immunization have been described (32). Thus, it would seem that antibodies produced by the *in vitro* and *in vivo* procedure are primarily directed against different classes of epitopes in nuclear proteins.

DISCUSSION

We have described procedures for stimulating lymphocytes *in vitro* with weakly immunogenic, insoluble antigens and for

Table 3. Dependence of the numbers of specific hybridomas on the concentration of antigen

Quantity of antigen, μg	Clones,* no.	Stimulated cells,† no.	Clones secreting antibody,‡ no.	Positive clones,§ %	Specific clones,¶ %	Subtype
sc-1						
0.05	43	1.2×10^6	10	60	0	IgM
0.1	68	4.6×10^6	17	76	0	IgM
1.0	59	6.8×10^6	12	75	0	IgM
2.5	93	5.1×10^6	18	67	15	IgM
5	87	6.9×10^6	21	76	12	IgM
M_r 210,000 nucleolar						
0.001	115	3.5×10^6	21	71	6	IgM
0.05	78	4.5×10^6	15	67	50	IgM
0.1	161	4.1×10^6	28	71	39	IgM
1	86	6.7×10^6	16	56	55	IgM
Vimentin						
0.001	79	4.6×10^6	23	65	67	IgM
0.1	98	8.6×10^6	17	53	77	IgM
1	119	1.07×10^7	31	81	64	IgM + IgG
Silica						
1	15	1.0×10^6	7	0	0	NT
10	26	0.9×10^6	5	0	0	NT
100	18	1.3×10^6	5	0	0	NT
500	6	0.8×10^6	0	0	0	NT
Cu^{+2}-NHCP						
1	67	5×10^6	17	65	0	IgM
10	88	5.8×10^6	19	47	0	IgM
50	61	4.0×10^6	23	69	0	IgM
100	72	8×10^6	28	54	6	IgM

Spleen cells from six BALB/c × c57/B1 F_1 mice ($5–6 \times 10^8$) were prepared and stimulated 8 hr in the presence of the quantities of antigen listed above coupled to 500 μg of silica. Media contained all of the additives described in Table 2. After growing 8 days, spleen cells were fused with PAI/O myeloma cells. Ig class was determined by Ouchterlony diffusion (31). NT, not tested.

*Number of estimated clones per fusion surviving past the 16-cell colony size.

†Number of nonreticulocyte spleen cells surviving 5–6 days in culture and excluding trypan blue.

‡Number of total clones secreting antibody.

§Percentage of original clones secreting antibody reacting in the dot-blot assay and/or immunofluorescence assay and, therefore, secreting antibody reacting with some protein derived from HeLa cells.

¶Percentage of positive clones secreting antibody that react via immunoblot assay specifically with respective antigen. In the case of Cu^{+2}-NHCP, the specific clones are those producing antibodies specific for sc-1.

the production of specific monoclonal antibodies. The protocol can be discussed in terms of five critical parameters. First, an induction period is used to define the repertoire of response of the capable lymphocytes. The *in vitro* priming occurs in the absence of serum and, therefore, reduces the possible problems of competition by serum components. It seems that only the most highly immunogenic antigens elicit a significant response in the presence of fetal calf serum. Second, the use of antigen coupled to fumed silica apparently allows appropriate presentation of antigen. Both soluble and insoluble antigens are readily and irreversibly bound to silica, and the resulting complex is easily dispersed into the stimulation reaction. The antigen–silica complex induces aggregation of lymphocytes and, thus, enhances the probable requirement of direct cell–cell contact and antigen binding (33, 34). Silica itself may act as a macrophage attractant or activator, thus triggering the putative requirement for macrophage involvement in T cell-mediated responses (35–38). Third, we have noted a requirement for seemingly high cell concentrations during lymphocyte stimulation (39), but we do not note a narrow range of cell concentrations critical for response. It seems probable that the initial silica-induced clustering of cells is responsible for this less critical requirement. Fourth, a complex set of anti-suppressor T-cell drugs and nonspecific growth factors is included during the stimulation period. We have not established whether the anti-suppressor drugs significantly expand the repertoire of the preimmune spleen, but the concept appears worthy of study. An absolute requirement for thymocyte-conditioned medium is shown, but autologous as well as heterologous thymocytes are capable of conditioning media and we have not adopted the use of mixed-thymocyte conditioning. In this procedure there exists no particular requirement for the use of preselected fetal calf serum for supporting lymphocyte division, which is probably due to the use of glutathione (40). Rabbit serum has been reported as a successful substitute for fetal calf serum. We have found allogenic and autologous sera to be inhibitory, which is in agreement with other findings (41). Fifth, we have critically reexamined the current paradigm that extraordinarily small quantities of antigen are required to elicit a response *in vitro* (3). The data presented appears to indicate that, although in some cases nanogram levels of antigen are immunogenic, different protein antigens, especially if conserved through evolution, require substantially higher quantities.

The response to antigen elicited *in vitro* is a primary re-

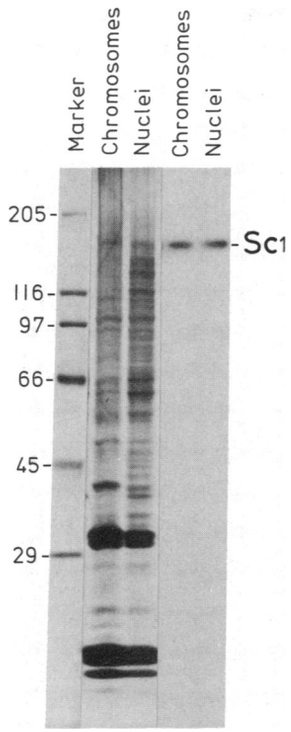

FIG. 1. Immunoblot analysis of anti-sc-1 antibodies. HeLa metaphase chromosomes and interphase nuclei were prepared, and proteins were isolated. Identical amounts of proteins were subjected to electrophoresis in gradient 7.5–15% polyacrylamide gels. (*Right*) Half of the gel was transferred to nitrocellulose and immunoprobed with an anti-sc-1 antibody and ^{125}I-labeled secondary antibody as described (11). (*Left*) The other half of the gel containing the same proteins was stained for visualization of total proteins. The positions of marker proteins (in kDa) and of the sc-1 protein of 170 kDa are indicated.

sponse; hence, we have immortalized primarily activities in the IgM subclass. It has been suggested that IgG-secreting hybridomas can be obtained by extending the incubation period to 6 or 7 days and restimulating the lymphocyte culture (2). We have been largely unsuccessful at attempts to obtain IgG-secreting hybridomas by extended culture but other protocols can be suggested.

Recently, preliminary results with this procedure in collaboration with Peter Brams and Karen Linnet in the laboratory of Lennart Olsson at the State University Hospital in Copenhagen have indicated that highly specific monoclonal antibodies were obtained against a mammalian core histone and a subunit of the insulin receptor. Thus, it appears this procedure is applicable to other insoluble and weak immunogens derived from both chromosomes and membranes.

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1. Reading, C. L. (1982) *J. Immunol. Methods* **53**, 261–291.
2. Pardue, R. L., Brady, R. C., Perry, G. W. & Dedman, J. R. (1983) *J. Cell Biol.* **96**, 1149–1154.
3. Luben, R. A., Brazeau, P., Bohlen, P. & Guillemin, R. (1982) *Science* **218**, 887–889.
4. Hoffman, M. K., Schmidt, P. & Oettgen, H. F. (1973) *Nature (London)* **243**, 408–409.
5. Hoffman, M. K. (1979) *Ann. N.Y. Acad. Sci.* **332**, 557–564.
6. Mishell, R. I. & Dutton, R. W. (1967) *J. Exp. Med.* **126**, 423–442.
7. Iscove, N. N. & Melchers, F. (1978) *J. Exp. Med.* **147**, 923–928.
8. Burger, M. (1977) *Eur. J. Immunol.* **7**, 906–908.
9. Click, R. E., Benck, L. & Alter, B. J. (1972) *Cell Immunol.* **3**, 264–269.
10. Lewis, C. D. & Laemmli, U. K. (1982) *Cell* **29**, 171–181.
11. Van Ness, J. & Pettijohn, D. E. (1983) *J. Mol. Biol.* **171**, 175–205.
12. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
13. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617.
14. Kenny, J. W., Lambert, J. M. & Traut, R. R. (1979) *Methods Enzymol.* **59**, 545.
15. Morrison, M., Bayse, G. S. & Webster, R. G. (1971) *Immunochemistry* **8**, 289–294.
16. Galfare, G. & Milstein, G. (1981) *Methods Enzymol.* **73**, 3–46.
17. Kohler, G. & Milstein, C. (1975) *Nature (London)* **256**, 495–497.
18. Gefter, M., Harguilies, D. H. & Scharff, M. D. (1977) *Somatic Cell Genet.* **3**, 331–339.
19. Burger, M. (1980) *FEBS Lett.* **118**, 212–214.
20. Mancino, D. & Ovary, Z. (1980) *Int. Arch. Allergy Appl. Immunol.* **61**, 253–257.
21. Burger, M. (1982) *Immunology* **45**, 381–385.
22. Haynes, B. F. (1981) *Immunol. Rev.* **57**, 127–161.
23. Ogden, D. E. & Hill, H. R. (1980) *Immunology* **41**, 107–114.
24. Osband, M. E., Hamilton, D., Shen, Y. J., Cohen, E., Schlesinger, M., Lavin, P., Brown, A. & McCaffrey, R. (1981) *Lancet* **i**, 636–638.
25. Black, J. W., Duncan, W. A. M., Durant, D. J., Ganellin, C. R. & Parsons, E. M. (1972) *Nature (London)* **236**, 385–390.
26. Merety, K., Room, G. & Maini, R. N. (1981) *Agents Actions* **11**, 84–88.
27. Cavagnaro, R. J. (1982) *Biotechnology* April, 32–41.
28. Tarc, M. J., Olsen, R. G. & Jacobs, D. L. (1982) *Immunopharmacology* **4**, 139–148.
29. Knapp, W., Berger, R. & Posch, B. (1982) *Immunopharmacology* **5**, 1–10.
30. Andersson, J., Coutinho, A., Lernhardt, W. & Melcheres, F. (1977) *Cell* **10**, 27–34.
31. Ouchterlony, O. (1949) *Acta Pathol. Microbiol. Scand.* **26**, 507–510.
32. Epstein, A. L. & Clevenger, C. V. (1984) *Recent Advances in Nonhistone Protein Research*, ed. Bekhor, I. (CRC, Boca Raton, FL), in press.
33. Pierce, C. W. & Benacerraf, B. (1969) *Science* **166**, 1002–1004.
34. Sulitzeanu, D., Kleinman, R., Benezra, D. & Gery, I. (1971) *Nature (London) New Biol.* **229**, 254–255.
35. Duclos, H., Galanaud, P., Mailbt, M. C., Crevon, M. C. & Dormont, J. (1979) *Scand. J. Immunol.* **9**, 159–168.
36. Morrissey, P. J., Boswell, H. S., Scher, I. & Singer, A. (1981) *J. Immunol.* **127**, 1345–1353.
37. Miner, K. M., Reading, C. L. & Nicholson, G. L. (1981) *Invasion Metastasis* **1**, 58–63.
38. Herngartner, H., Luzzati, A. L. & Schreier, M. (1978) *Curr. Top. Microbiol. Immunol.* **81**, 92–97.
39. Knight, S. C. (1982) *J. Immunol. Methods* **50**, R51–R63.
40. Hoffeld, J. & Oppenheim, J. J. (1980) *Cell Immunol.* **53**, 325–327.
41. Mann, P. L. & Falk, R. E. (1972) *Proc. Leucocyte Cult. Conf.* **7**, 39–41.