

receptor ligand conjugated to BSA will induce the production of anti-idiotypic antibodies to the combining sites of the antigen specific antibodies. The great advantage of this approach is that no purified receptor is required.

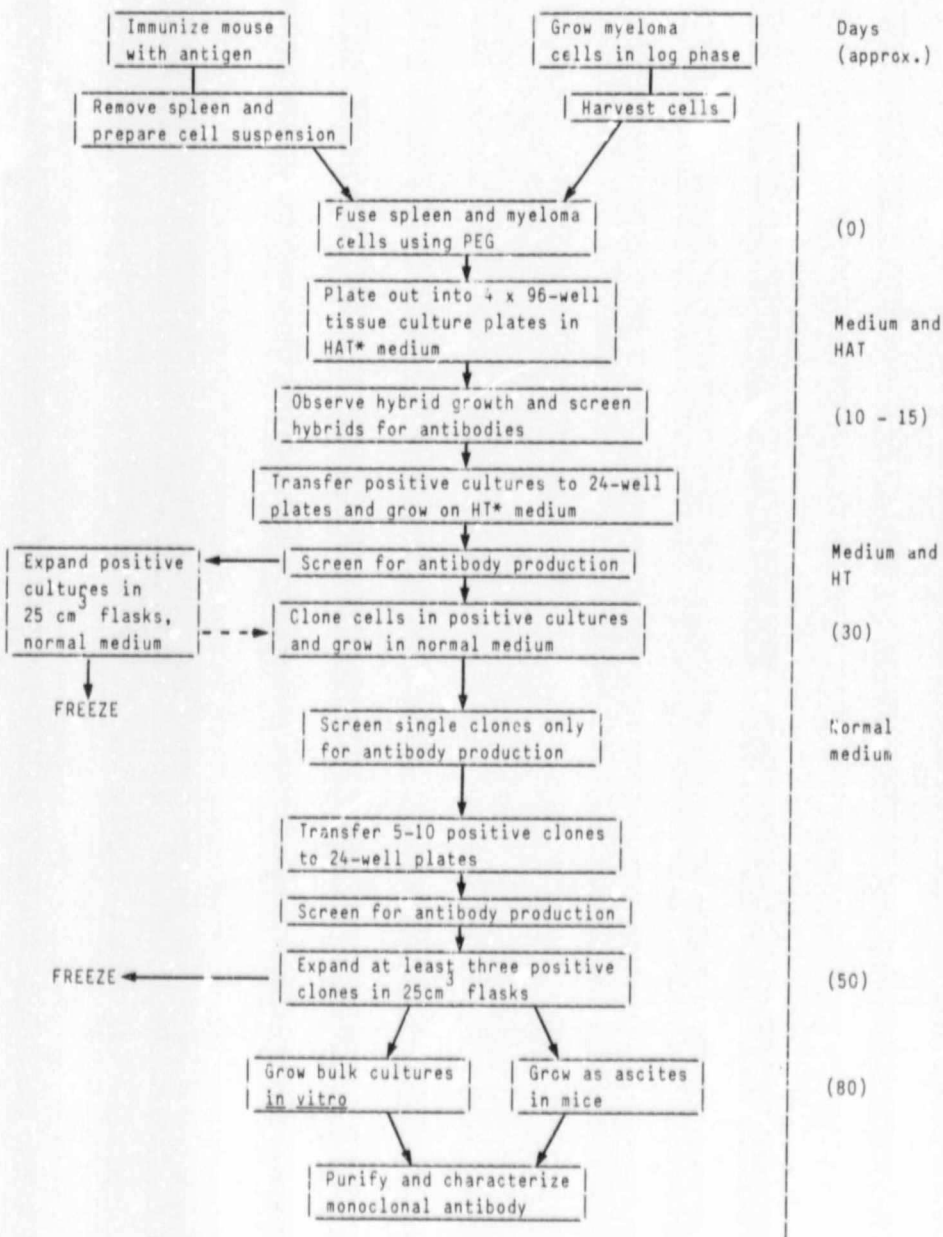
4.3.3 Fusion, selection and maintenance of hybrids

A number of fusion protocols have been documented using polyethylene glycol (PEG) as fusing agent (210, 215, 216). In addition to PEG, dimethyl sulphoxide (DMSO) is sometimes employed to improve fusion (217), although some authors reported a minimal effect with DMSO (215). A mixture consisting of a 10 : 1 ratio of spleen to myeloma cells is commonly used during fusions (209). The different stages during McAb production is summarized in Scheme 4.1 (reproduced from reference 209).

Spent medium from the polyclonal hybridoma cell cultures was assayed for antibody activity against partially purified HEBP (male and female) and uterine cytosol. From a total of 474 wells, 34 gave positive results against female HEBP (♀ - HEBP), 8 against male HEBP (♂ - HEBP) and one reacted positively against all three antigen preparations. These results are summarized in Table 4.3. Thus a 9% positive reaction against the partially purified HEBP was obtained.

Enrichment of the spleen cell culture for the B cell which is making the desired antibody may be a solution for the problems experienced in obtaining specific McAb from animals immunized with a weak immunogen or minute amounts of the antigen. French et al. (213) reported that a 50-fold increase in hybridomas making the desired antibody, compared to a standard fusion, was obtained when spleen cells harvested for fusion have been re-injected into irradiated recipients along with the antigen. Another approach to enrich the cells making the desired antibody is to immunize the spleen cells in vitro using spleen cells obtained from immunized animals or as a primary

SCHEME 4.1: PRODUCTION OF MONOCLONAL ANTIBODIES (TAKEN FROM REFERENCE 209)



*HAT = Medium containing hypoxanthine, aminopterin and thymidine.

*HT = Medium containing hypoxanthine and thymidine.

TABLE 4.3: RESULTS OBTAINED DURING THE ELISA EXECUTED ON THE SPENT MEDIUM OF POLYCLONAL CELL CULTURES.

Preparation used to coat microtiter plates	Reaction of spent medium from three types of Polyclonal cell cultures (% of total number of polyclonal cell cultures)		
	^c A (0,2)	B (7,2)	C (1,7)
^a VMUC	+	-	-
^b HEBP (♀)	+	+	-
HEBP (♂)	+	-	+

^a VMUC = vervet monkey uterine cytosol.

^b HEBP used for coating was partially purified.

^c A, B, C = different groups of polyclonal cell cultures.

immunization (213). Antibody producing hybridomas can be generated in this manner when only small amounts of the antigen is available. Thus, the hybridoma technique makes it possible to obtain McAb even from a weak immunogen available in small amounts.

4.3.4 Screening methods

The need for a screening procedure which is fast, reliable, sensitive and relevant, prior to fusion of cells, has been stressed by numerous workers (209, 210). With a proper screening assay, useful hybrids should be selected as early as possible to selectively clone the relevant hybrids. Cloning and freezing in of positive clones are time consuming steps in the process and it is therefore important to select only the most useful hybridomas. The high level of competition for growth between different clones in a polyclonal cell culture together with chromosome segregation that leads to instability of expression are very important reasons to clone and reclone as early as possible. Some care must be taken when developing a screening assay to detect McAb. Firstly the concentration of antibody in the culture filtrate is approximately 1 - 25 $\mu\text{g}/\text{ml}$ compared to that of the hyperimmune serum 0,1 - 10 mg/ml (209). Immunoassays often rely on the polyvalent recognition of antigens as obtained by polyclonal antisera (209).

Antibody-secreting hybrids can be identified by the detection of antibody in the spent medium (binding, hemagglutination and lytic assays together with assays based on biological activity of the antigen) or the direct detection of antibody-secreting cells (plaque-forming clones) (210). Binding assays are markedly suitable for McAb work because they are highly specific, sensitive, simple and could be applied for all classes of antibodies and most antigens. The indirect binding solid phase assay and immunoprecipitation were two binding assays still under investigation for employment as screening

assays during the McAb production against the HEBP. In solid phase assays like ELISA, the antigen is bound to a solid support (plastic, filter paper, Sepharose, nitrocellulose). Antibodies in the tissue culture supernatants are bound to the antigen and measured by binding a second labelled (radio, enzyme or fluorescent) antibody. Although the use of a radio labelled second antibody is highly sensitive, an enzyme linked second antibody which excludes radioactive hazards, is inexpensive, stable and provides a fast visual result. Variation on the ELISA includes the use of a radioactive substrate to measure the second antibody-enzyme conjugate (218) or the use of radiolabelled protein-A instead of a secondary antibody (219). One should, however, note that the latter compound doesn't bind to Fc regions of all classes.

During immunoprecipitation the culture supernates are screened for their ability to precipitate a labelled protein (antigen) when a second antibody or protein-A is added. A pure iodinated or tritiated antigen must be used during this assay, unless the precipitate is to be analysed on a polyacrylamide gel (209). A pure antigen could not be employed in the immunoprecipitation assay employed during the production of McAb to the HEBP. However, distinction between specific and non-specific proteins was made possible by the addition of a 1 000-fold excess of unlabelled DES during the preparation of the radioactive hormone-receptor complex (Section 4.2.5). The immune complex obtained following incubation of the hybridoma culture supernates with immune and control sera should be precipitated by a second antibody using a quantity that gives maximum precipitation. Chicken anti-mouse Ig (IgY) and normal mouse serum were standardized in order to determine the optimal amounts required to give maximum precipitation of the immune complex with the minimum amount of IgY (Table 4.4 and Figure 4.3). Results obtained during screening of immune and control sera with the ELISA and immunoprecipitation assays are illustrated in Figures 4.1 and 4.2 and summarized in Table 4.2.

TABLE 4.4: A SUMMARY OF THE RESULTS OBTAINED DURING THE QUANTITATIVE PRECIPITATION REACTION BETWEEN NORMAL MOUSE SERUM (11 $\mu\ell$) AND DIFFERENT VOLUMES (5 - 35 $\mu\ell$) OF CHICKEN ANTI-MOUSE IMMUNOGLOBULIN (IgY).

IgY ($\mu\ell$)	Normal mouse serum ($\mu\ell$)	PBS ($\mu\ell$)	^a Protein Content		
			Supn. (μg)	wash (μg)	Ppt. (μg)
5	11	984	472,9	2,57	1,38
10	11	979	492,3	2,63	1,60
15	11	974	519,1	3,23	2,17
20	11	969	514,3	3,41	3,18
25	11	964	459,8	3,29	6,32
30	11	959	508,6	2,66	4,68
35	11	954	517,1	2,69	4,73

^a The protein content of the different samples was determined by the method of Bradford (152) as described in Section 3.2.10.

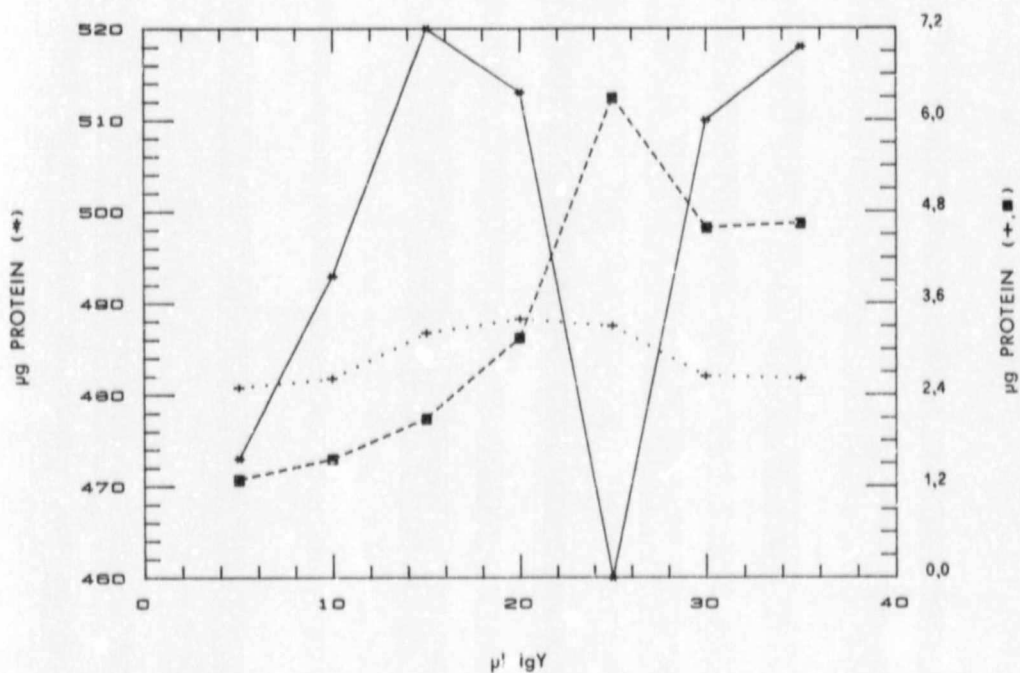


FIGURE 4.3

The quantitative precipitation reaction between normal mouse serum and chicken anti-mouse Ig (IgY). A constant volume of normal mouse serum (11 µl) were added to different quantities of IgY and incubated for 5 h at 4°C. Following centrifugation and washing with PRS the protein content of the supernatant (*—*), wash (+...+) and precipitate (■--■) was determined.

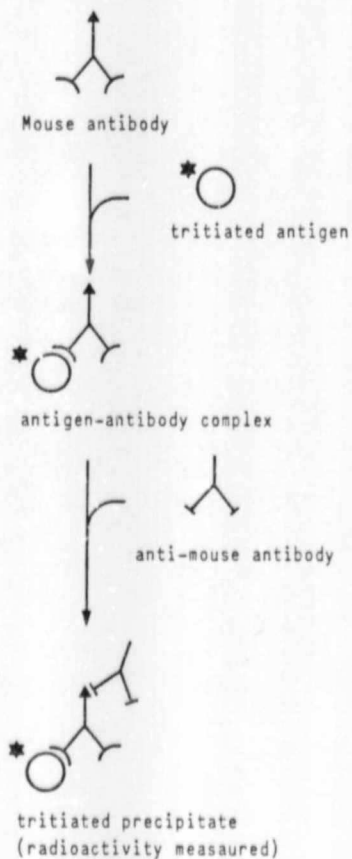
In Scheme 4.2 a comparison between immunoprecipitation and ELISA is illustrated. The double antibody precipitation assay for the detection of antibodies against the oestrogen receptor (204, 205, 212) appears to be the assay of choice in many laboratories. However, during the production of McAb to the HEBP, no positive reaction was obtained with this assay in contrast to the ELISA. Other disadvantages of the immunoprecipitation assay are that it is more laborious (220). The ELISA used for the detection of antibodies to the HEBP is a reliable and fast immuno assay, but it has numerous limitations. In this assay no distinction can be made between antibodies produced against specific and non-specific HEBP. Thus a great deal of the positive reaction observed during screening with the ELISA could be the result of antibodies produced against contaminants present in the partially purified HEBP preparation, used for immunization and microtiter plate coating.

4.3.5 Cloning

Results obtained for the screening of the monoclonal cell cultures for antibody activity against different antigen preparations are summarized in Table 4.5. Three polyclonal hybrid lines (A, B and C) were cloned. The antigen preparations (used for coating of the microtiter plates) against which these lines have shown antibody activity prior to cloning, are listed in Table 4.3. Polyclonal hybrid line A has shown antibody activity against all three antigen preparations (uterine cytosol, partially purified HEBP from male and female vervet monkey). This hybrid line was therefore more important and was subsequently cloned twice with a large number (96 and 120 respectively) of picked clones. The two other polyclonal hybrid lines (B and C) have shown antibody activity against one antigen preparation respectively. Cloning of a B-type polyclonal hybrid line was done once with 24 clones picked while two C-type hybrid lines were cloned and

SCHEME 4.2: A COMPARISON BETWEEN IMMUNOPRECIPITATION AND ELISA AS SCREENING METHODS TO DETECT ANTIBODY, PRESENT IN IMMUNE SERUM, AGAINST THE APPROPRIATE ANTIGEN.

Immunoprecipitation



ELISA

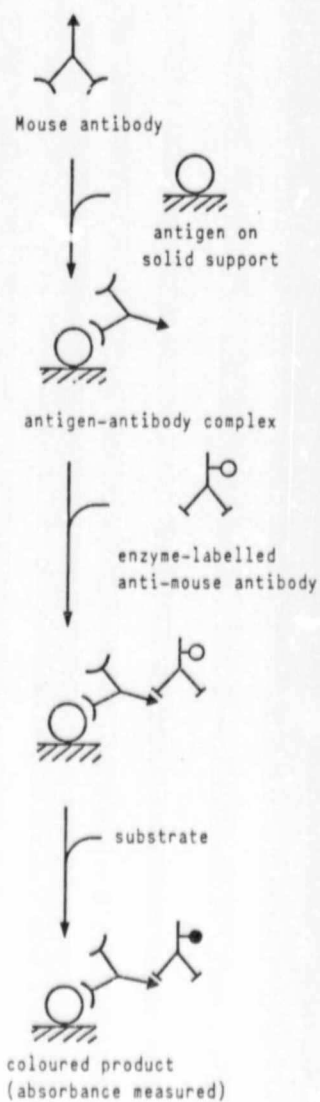


TABLE 4.5: A SUMMARY OF THE ELISA RESULTS OBTAINED DURING CLONING OF THE POLYCLONAL CELL CULTURES WHICH SHOWED A POSITIVE REACTION AGAINST DIFFERENT ANTIBODY PREPARATIONS.

	Postive controls	Polyclonal cell cultures			
		A	B	C	
Number of clonings done	:	2	1	2	
Number of clones picked	:	96	120	24	24 24
Antibody reaction against					
^a antigen preparation	:				
^b VMUC	:	-	-	-	- -
^c HEBP (♂)	:	-	-	-	- -
^c HEBP (♀)	:	-	-	-	- -
^d UEBP	:	-	-	-	- -

^a Antigen preparations used for coating of the microtiter plates.

^b VMUC = vervet monkey uterine cytosol.

^c HEBP = partially purified hepatic oestrogen-binding protein.

^d UEBP = partially purified uterine oestrogen-binding protein.

24 clones selected with each cloning. A total of 288 monoclonal cell cultures were screened for antibody activity against four antigen preparations with the aid of the ELISA. No antibody activity against the four antigen preparations (Table 4.5) could be detected in the spent medium of any of the screened monoclonal cell cultures.

The development of a fast, sensitive and specific screening assay was seriously hampered by problems encountered in purifying the HEBP to homogeneity, with the aid of affinity chromatography. The need for an assay that can specifically identify the clones producing the desired antibody can not be over-estimated. The ELISA used in this study did not provide absolute specificity that resulted in the selection of a large number of positive cultures that could not be conveniently handled. Instability and clonal competition are particularly frequent in early stages of growth (209). Thus, the cloning of positive polyclonal cell cultures must be done as early as possible. Considering the fact that the selected polyclonal cell cultures were maintained for nearly two months prior to cloning, the above-mentioned problem could have been the main reason for the failure in obtaining the desired McAb. Other aspects that should have received greater attention were the regular screening of supernatants and the preparation of stocks of clones (freezing) before the culture became negative. Cloning can be done either in semi-solid agarose or by limiting dilution (221). Agarose is known to cause a lower cloning efficiency and this may result in the selection of vigorous but unwanted hybrids. Colonies of cells picked from agar are often not absolutely pure clones (210).

4.4

SUMMARY

A 6091-fold purified HEBP was injected into three C57 black/6 mice (both sexes). Antibody activity could positively be identified in two of the three immune sera by an ELISA, seven days after a booster injection. The mice were given a final

injection of the antigen three months later and sacrificed. Sp^{2/0} myeloma cells were fused with the mouse spleen cells (1 : 10) and plated out in 474 wells of microwell tissue culture plates. Antibody activity was detected by ELISA in 43% of the polyclonal cell culture supernates. The polyclonal cell cultures were cloned on agar and 288 clones selected. No antibody activity could, however, be detected in the spent media.

CHAPTER 5

CONCLUDING DISCUSSION

Over the past decade hepatic oestrogen-binding proteins have been demonstrated in a variety of species (103, 146, 147, 154). In some cases sex related differences have been illustrated (104, 106, 149). Male and female vervet monkey liver cytosol contains the same levels of high affinity oestrogen-binding proteins (Table 2.4). With the aid of multipoint saturation analyses an additional binding protein, exhibiting a high capacity and moderate affinity was identified in the liver cytosol from male vervet monkeys (Figures 2.6). SDG analyses, however, revealed the presence of two binding components (3,8S and 8,1S) in both sexes (Figure 2.7). A male-specific binding protein (male-SBP) (104), an unusual oestrogen-binding protein (UEBP) (106) and a high capacity, low affinity (HCLA) oestrogen-binding protein (112) have been described in the literature. This protein reportedly exhibits moderate affinity, a high capacity and sediments at 3-4S on SDG and can not be precipitated at 30% ammonium sulphate saturation (104, 106). According to the supporters of the male-specific binder, this protein is present in very low levels in the female liver but features prominently in the adult male liver (104, 106, 112). The data obtained in this study illustrated that the high capacity, moderate affinity binding protein was more abundant in the male monkey liver although it was present in significant amounts in the female liver (Figures 2.6 and 2.7). In this study it is clearly shown that this binder is not an exclusive property of the adult male liver.

The role of the molybdate oxyanion in the stabilization of steroid hormone receptors features as an important topic over the last decade (155-159). It was previously postulated that the interaction of the molybdate oxyanion with the receptor might cause a positive cooperative effect on oestradiol binding. However, the opposite effect with the HEBP was observed (Table 2.5, Figure 2.8). This discrepancy might be the result of negative cooperative binding due to conformational changes in the HEBP or differences in the molecular properties of the hepatic and uterine

oestrogen-binding proteins. From the SDG profiles the 8,1S binding component appeared to be stabilized in the presence of molybdate at the cost of the 3,8S component (Figure 2.8).

Corticosteroid binding globulin (CBG) displays some affinity for oestrogens and is furthermore, abundantly present in the liver (160, 161). A possible contribution of hepatic CBG to the high levels of binding activity in the liver has therefore been investigated. However, the presence of a large excess of hydrocortisone during titration assays and SDG analyses did not have any effect on the levels of HEBP measured (Table 2.5, Figure 2.8).

Two specific binding components were observed with GPC of the HEBP from male and female vervet monkeys (Figures 2.9 and 2.10); 4×10^5 daltons and 4.2×10^4 daltons. High ionic strength conditions favour the 4.2×10^4 dalton component and more so the female 4.2×10^4 dalton component. Thus, sex related differences in vervet monkey hepatic oestrogen-binding protein levels and some molecular properties appear to be either absent or very small. Considering the result obtained during this investigation it can be inferred that the 8,1S oestrogen-binding protein is a typical oestrogen receptor and that in addition to this component there is a second, low affinity oestrogen-binding protein present in livers of both sexes. However, this binder reaches higher levels in the male than in the female monkeys.

In the effort to purify the HEBP from the vervet monkey, nuclear extraction and salt fractionation were initially examined as prepurification methods. However these methods were found to be insufficient for the purification of the HEBP (Tables 3.1 and 3.2). In contrast with these results Gschwendt et al. (172) reported the purification to homogeneity of the nuclear ER from chick liver by using a combination of ammonium sulphate precipitation and affinity chromatography.

Heparin, a highly sulphated glycosaminoglycan, dissociates aggregates of oestrogen-receptor complexes via a mechanism not exactly understood (177).

Heparin-Sepharose chromatography is a qualifying step during purification of the HEBP for the following reasons: (1) it allows partial purification of the HEBP (Table 3.3), (2) it serves as a concentrating procedure (Table 3.8), (3) the heparin used for the elution of protein adsorbed onto the heparin-Sepharose affinity matrix, prevents aggregation of the receptor and (4) heparin-Sepharose chromatography eliminates the excess unlabelled ligand employed during elution of specifically adsorbed protein from DES-Agarose; the preceding step (Table 3.7). The HEBP, partially purified by heparin-Sepharose chromatography (Figure 3.2) exists as two entities with sedimentation coefficients of 3,8S and 8,1S respectively (Figure 3.3). The latter is less prominent under high ionic strength conditions.

Specific affinity chromatography (DES-Agarose) proved to be the most powerful tool during the partial purification of the vervet monkey HEBP (Table 3.5, Figure 3.4). DES-Agarose binds oestradiol-binding proteins selectively with high capacity (173). The spacer arm between DES and agarose is stable and therefore not susceptible to cleavage by cytosolic enzymes. Derivatization of the matrix or ligands is not required for the covalent coupling reaction. Thus, the preparation of DES-Agarose is relatively easy. An enrichment of the vervet monkey HEBP of approximately 7 435-fold (30% recovery) was achieved following DES-Agarose and heparin-Sepharose affinity chromatography (Table 3.8), which compares favourably with the partial purification values reported for the chicken liver ER (168). The apparent loss of HEBP may be attributed to three factors: (1) non-specific interactions of the HEBP with the affinity matrix that result in the loss of this protein during the washing procedures (174), (2) the decreased stability of the protein when it is highly purified (174) and (3) the incomplete elution of specifically adsorbed HEBP from the affinity matrix (164).

The observed isoelectric values for the partially purified vervet monkey HEBP (6,8 and 6,9) correlate with those of 6,6 and 6,8 found for the calf uterine oestrogen receptor (170) and with those of 6,8, 6,2 and 5,9 reported for the vervet monkey uterine receptor in the absence of sodium molybdate. Various molecular masses for the isolated oestrogen receptor have been reported. Following SDS-PAGE of purified rabbit uteri ER, Van

Oosbree et al. (173) observed two oestrogen-binding proteins with molecular masses of 50 000 and 65 000 daltons. The purified chick liver ER of Gschwendt et al. (172) migrated as a single band on a SDS polyacrylamide gel with a molecular mass of 55 000 dalton. During this study clear evidence for three HEBPs were found with molecular masses of 30 000, 38 000 and 68 000 daltons.

For the preparation of monoclonal antibodies the purity of an immunogen employed during immunization is irrelevant if the method of assay distinguishes between antibodies to the specific component and antibodies to the impurities (210). The assay used during screening stages to detect and clone the hybrid secreting the desired antibody is the most important factor and should be given a great deal of attention (209, 210).

The vervet monkey HEBP, purified 6 091-fold (Table 4.1) with DES-Trisacryl GF 2000 affinity chromatography, was employed as immunogen. Following an immunization program and screening of immune sera (Figures 4.1 and 4.2), spleen cells of the immunized mice were fused with Sp^{2/0} myeloma cells. A 43% positive reaction (ELISA) of the polyclonal hybridoma cell cultures against the antigen was obtained (Table 4.3). The double antibody precipitation assay (Scheme 4.2) for the detection of antibodies against the ER appears to be the assay of choice in many laboratories (105, 205, 212). However, this assay was found to be insufficient during this investigation (Figure 4.1 and Table 4.2). It was therefore decided to employ the ELISA (Scheme 4.2) while solving the problem with the more specific double antibody immune precipitation assay. Screening methods should, however, be well worked out before cell fusion (209). Since the problems experienced with the double antibody precipitation assay was not cleared up at the time the cell fusion was executed, the ELISA was employed as screening assay. However, this assay did not provide absolute specificity and a large amount of non-specific hybridoma cultures were therefore detected. The positive polyclonal cell cultures were cloned and a total of 288 clones selected. Unfortunately no antibody activity could be detected in the spent media of any of the monoclonal cell cultures (Table 4.5).

In conclusion, the main reasons responsible for the unfortunate outcome of this part of the study may be the following: (1) instability and clonal competition between polyclonal cell cultures that are particularly frequent in early stages of growth (209, 210), (2) the relatively long period preceding cloning of positive polyclonal cell cultures and finally (3) the insufficient specificity of the screening assay that resulted in a large number of positive cultures detected which could not be conveniently handled.

* * *

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