REVISTA ESPAÑOLA DE FISIOLOGIA, 40, 279-288. 1984

High Stability Hybrids Producing Monoclonal Antibodies Against Human C-Reactive Protein

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(Received on October 10, 1983)

M. ITURRALDE and J. COLL. *High Stability Hybrids Producing Monoclonal Antibodies Against Human C-Reactive Protein.* Rev. esp. Fisiol., **40**, 279-288, 1984.

This report shows how high stability hybrids producing monoclonal antibodies against human C-reactive protein were raised and selected. Monoclonal antibodies can be produced in large enough quantities through this method, to allow for the design and use of quantitative C-reactive protein determination on a clinical scale. This novel strategy consisted of the following: growing the hybrids and freezing them before cloning in order to assure stability and selecting the hybrids from those producing high titres in mouse ascite induction. Two monoclonal antibodies of high stability and great potential for large scale production have been developed in this manner.

Production on a large scale of these monoclonal antibodies against human C-reactive protein can be useful both in clinical quantification and in physiological studies concerning its still unknown *in vivo* function.

Key words: C-reactive protein, Monoclonal antibodies hybrids.

C-reactive protein (CRP) is the prototype of acute phase proteins. Following most forms of tissue injury, its plasma level increases by as much as 1,000 times from a normal value of 1-2 μg/ml within hours of onset of most tissue damaging inflammatory processes. CRP undergoes calcium dependent binding to phosphoryl choline residues of phospholipids, and once the complex is formed, it activates the classical complement pathway (8). CRP has also been reported to enhance phagocytosis, inhibit platelet function and antigen-induced T-cell activation. However, despite this *in vitro* activity, its role *in vivo* is still uncertain (8).

A number of different diseases are characterized by high circulating levels of CRP (8). When clinical symptoms and laboratory indices of disease activ-

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ity are reduced either spontaneously or following treatment, the CRP levels fall. CRP is the single most sensitive objective criterion of disease activity. The close correlation between disease activity and CRP level provides valuable information for treating patients. Therefore, the monitoring of healthy individuals could be used in the detection of early forms of cancer.

The measurement of serum CRP levels is also of value in distinguishing pyelonephritis from cystitis, bacterial pneumonia from acute bronchitis, bacterial from aseptic meningitis, and systemic lupus erythematosus from rheumatoid arthritis. Interpretation of CRP data requires careful quantitative assessment as well as serial sampling.

CRP was initially detected and assayed through its reaction with C-polysaccharide. Current clinical tests include precipitation, radial immunodiffusion, passive agglutination of latex particles, etc. However, these methods are only semi-quantitative. For clinical purposes, a suitable method to measure serum levels in the range of 1 to 600 μg/ml, that is rapid, precise and reproducible for automation is needed. In order to develop an ELISA with all of these properties, and also provide anti-CRP for physiological studies, high stability hybrids producing monoclonal antibodies against human CRP have been developed, and large scale production has been initiated. This is only one example of the numerous applications that monoclonal antibodies are beginning to have in the field of medicine (1, 4).

Materials and Methods

CRP Purification. Human CRP was purified from ascitic fluid assayed by immunodiffusion against Difco anti-CRP. The liquids were then precipitated with $(NH_4)_2SO_4$. The pellet formed between 50 % and 75 % saturation was redissolved and dialyzed overnight against 0.02 M Tris-HCl, 0.01 $CaCl_2$ and 0.1 M NaCl pH 8. This fraction was added at 10 ml/ml to an agarose column 7 × 1 cm with immobilized 2 aminoethyldihydrogen-phosphate (6). The retained fraction was eluted from the column using 0.02 M Tris-HCl 0.02 M sodium citrate and 0.1 M NaCl pH 8 (13). 15 % polyacrylamide gel electrophoresis in 0.1 % SDS was used in determining purity. Only one single staining band was visualized at about 25 K, when using the silver nitrate staining method of Biorad. The proteins were measured by the Bradford reagent (2).

The methods used to measure CRP were as follows: double immunodiffusion on agar plates (6) and on ELISA-sandwich using polyclonal anti-CRP (anti CRP bound to polyvinyl plates reacting with the tested CRP and developed by polyclonal anti CRP labelled with horseradish peroxidase) to be described elsewhere (10).

Myeloma cell culture. Non-secreting myeloma SP2 (6) was used in these experiments. The cell culture was carried out in Sterilin bottles, gassed in air with 5 % CO_2, in a Heraeus incubator at 37° C. The culture medium used was DMEM, 15 % Fetal Calf serum, 15 mM Hepes, 15 mM sodium bicarbonate. Fresh glutamine 2 mM was added weekly and the medium was changed every 2-3 days depending on the cell density. Number of cells was estimated either by Tryptan blue exclusion or by counting under a phase contrast microscope with an eye-piece. Freezing in liquid N_2 was as described (6).

Hybridoma production. Mice were given 1 intraperitoneal injection with partially purified CRP, equivalent to 200 μg in Freund's coadjuvant (Difco).

One month later the mice were given 3 intravenous injections with 10 μg of CRP at 7 day intervals. Three days after the last injection, the spleen was removed and spleen cells were used for fusion as described (6, 11). In order to obtain polyclonal anti-CRP after the immunization protocol described, the mice were injected intraperitoneally with 10×10^6 myeloma cells. Ten days later anti-CRP was found to be concentrated in the ascitic fluid.

After optimization of the variables affecting hybrid formation (5), the following fusion protocol was selected for being both highly efficient and reproducible. 250 μl of PEG 1000 (Koch-light, Colnbrook) 40 % in DMEM 10 mM bicarbonate was added to a mixture of 10^8 spleen cells and 10^7 myelomas. After being constantly agitated for 3 minutes, the mixture was resuspended in a total of 9 ml of DMEM and immediately centrifuged at 400 g (5). The pellet was resuspended in DMEM 15 % FCS at 5×10^5 myeloma cells/ml in Sterilin bottles, and incubated at 37° C, 5 % CO_2. After one day of incubation, HAT containing medium was added for hybrid selection. After 7-10 days, the surviving cells were changed to HT containing medium, and later to normal medium. Cloning was as described (3, 6, 9, 11).

Assay of monoclonal antibodies. Anti-CRP secreting clones were detected on CRP-coated 96 well polyvinyl plated using an ELISA assay (6, 10). CRP dissolved in 2.5 mM $CaCl_2$, 0.25 mM Hepes, pH 7 was dried overnight at 37° C, 0.25 μg/50 μl/well. The plates were kept dry at 20° C. Before use they were washed twice with 0.2 mM borate, 0.075 M NaCl, 2.5 mM $CaCl_2$, 0.05 % Tween 80, pH 7.5. The supernatants were then added to the 50 μl/well, and after 1 h at 37° C were washed with the buffer. Bound anti-CRP anti-bodies were detected by incubation with peroxidase labelled anti-mouse IGg (Sigma) for 30 minutes. After additional washing the enzyme was measured with OPD as described (6); then after 30 minutes of enzyme reaction stopped with 3 N H_2SO_4, the activity was referred to as OD 450. Mouse plasma and ascite activity were always measured at 1/50 dilution in the cell culture medium. Plastic surfaces were avoided during pipetting.

Scale up of monoclonal production. After cloning, the CRP-positive hybridomas were grown in DMEM, supplemented with 15 % Fetal Calf Serum (Gibco, Grand Island, N. Y.). They were later grown as ascitic tumors in Balb/C mice, previously primed with 0.5 ml pristane (Aldrich Chemical Co., Milwaukee, Wisconsin). Ascitic fluid was drained directly or by prior intraperitoneal injection of 5 ml physiological saline solution (0.8 % NaCl). Blood was taken from the tail vein. About 200 μl was collected at each bleeding, put into heparinized kimble glass tubes and centrifuged immediately. Plasma was used for anti-CRP determinations.

The solid tumors that appeared were excised and broken up in 15 % FCS DMEM with the help of a Potter. They were washed twice by centrifugation at 400 g for 10 min, and then frozen or injected into the mice for ascite induction.

Results

Fusion and cloning. Figure 1 shows the typical development of a cell fusion experiment. Immediately after fusion, the cells were distributed among 4 Sterilin bottles at a theoretical density of 0.5×10^6 myeloma cells/ml, corresponding to about 0.1×10^6 live cells/ml, according to the estimations made after fusion (0.2×10^6 by phase

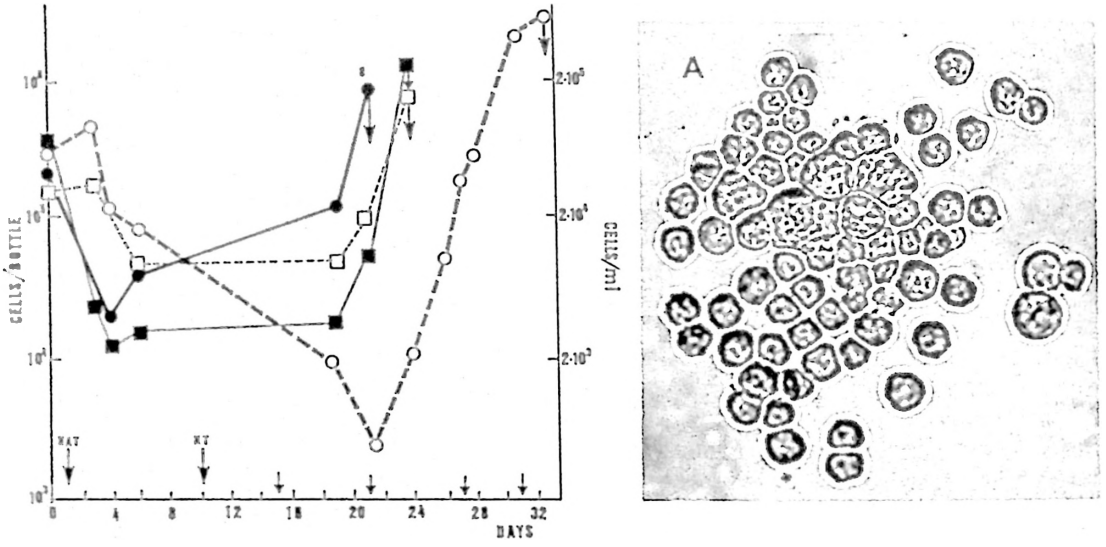

Fig. 1. *Cell evolution after fusion and hybrid selection.*
After fusion, the mixture of myeloma-spleen cells was distributed among 4 Sterilin bottles of 25 cm^2 in 5 ml of DMEM, 5 % FCS, 15 mM Hepes, 15 mM sodium bicarbonate, 2 mM fresh glutamine and 5 μg/ml gentamicine. After one day at 37° C in 5 % CO_2, HAT + 1/2 HT medium was added and the bottles were left in culture for 10 more days. On the 10th day, medium was changed to HT and on the 15th day to normal DMEM medium. The change of medium is indicated by the arrows in the figure. The A photo micrography represents the aspect of some clones growing after 10 days in culture.

contrast and 0.06×10^6 by Tryptan blue exclusion). The initial decrease in the number of cells, followed by phase contrast during the first week after fusion was due to the HAT effect on the no hybrids. However, as early as 3-4 days, small colonies (fig. 1) could be seen growing in this medium. Between 20 and 30 days, the cells were growing and then frozen in liquid N_2. Of the 4 bottles, the contents of 2 died after growing for 2 more weeks; the hybrids from the bottle with the fastest growth were selected for further study.

The cloning procedure was set up in three steps. Step I consisted of growing the hybrids at 600 cells/ml in 96 well plates using 100-200 μl of medium/well. After letting them grow for 5-7 days, the supernatants were assayed for the presence of monoclonal anti-CRP by the ELISA method already described (*Materials and Methods* and 6, 10). The results of one of these assays is shown in figure 2. Only those values $\geq$ 0.2 OD 425 were taken into consideration for further analysis. At this point, they were grown in 24 well plates (2 ml) and frozen to prevent losses. Step II was carried out by plating the hybrids of the previously selected wells at 3-10 cells/well in 96 well plates, as done beforehand. The selected wells were the A-9, A-8, A-7, B-7 and C-3. Each was distributed among 16 wells (figure 2). Five days after seeding, the number of cells present in each well was counted with an inverted microscope. This provided an early estimation of the number of clones/well. Twenty days after seeding and 3 days after the last change of medium, the supernatants were assayed for the presence of anti-CRP. Figure 2 shows the results of one such experiment. From the A-9, no positive cultures were obtained; from A-8, 4

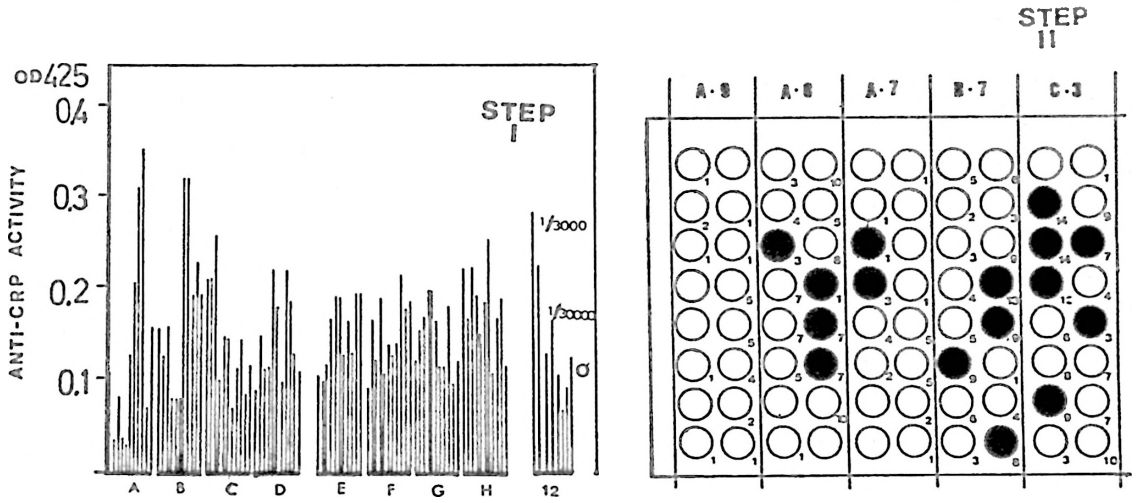

Fig. 2. *Screening of HAT resistant hybrid cloning.*
Cloning was performed in three steps: growing the cells from bottle number 6 (fig. 1) with 600 cells/well (step I); growing the positive cultures from step I with 3-10 cells/well (step II); and recloning the resulting positive hybrids in a semi-solid medium made by fibrin (9). Only the cultures with ≥ 0.2 OD 425 were considered to be positive. Step II represents the ELISA results, by showing positive hybrids as black circles, and negative hybrids as white circles. The number beneath the circles is the estimated number of cells 5 days after seeding. Positive control mouse serum with anti-CRP activity was measured at a 1/3,000 and 1/30,000 dilution.

positive cultures were obtained, one of which was monoclonal (D-4). From A-7, 2 positive cultures were obtained, one of which was monoclonal (C-5). Four positive cultures were obtained from B-7, and six from C-3. All the positive cultures were amplified in 24 well plates. After 1-2 weeks, a sufficient amount of cells from all of these clones were obtained for freezing and injecting into mice. Step III recloning was done by the fibrin semisolid method (3, 9). A total of 18 clones were selected for scaling up.

With the supernatants of these cultures, several preliminary results were obtained by using probes to assure the specificity of anti-CRP activity. Those included were: ELISA assay reproducibility (with different CRP preparations, different anti-IgG mouse labelled developers, repetition on different days, etc.), detection of mouse IgG in the supernatants, positive competition with CRP, and direct correlation between the amount of CRP bound to the ELISA plates and the result of the ELISA (data not shown).

Scaling up. In order to produce large amounts of hybrids, all the clones whose supernatants were positive *in vitro* were injected into pristane mice in small amounts (about $0.1 / 10^6$ cells/mouse) and examined two months later for anti-CRP in plasma and ascites. Control mice were injected with culture medium without cells. Figure 3A shows the anti-CRP activity of the clones selected. Mice designated with the numbers 3 and 11 developed solid tumors, but with very low anti-CRP activity.

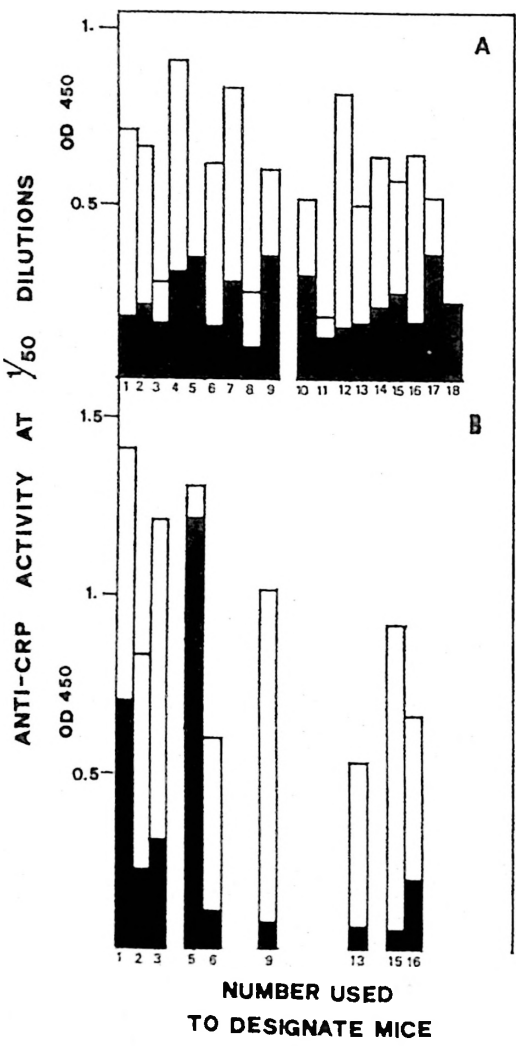

Fig. 3. *Anti-CRP made in mice injected with increasing amounts of hybrids.*

The scaling up of antibody production was done in two steps. First, about 100,000 cells from positive cultures were injected intraperitoneally into pristane primed mice. They were examined 2 months later for anti-CRP activity in plasma, 5 ml of ascites were induced and both were tested by ELISA assay (A). For further study, the hybrids in B were selected because of having the greatest amount of activity *in vivo*, good growth *in vitro* and the smallest amount of estimated clones. Second, 1-5 × 10^6 cells from the selected hybrids grown *in vitro* were injected into mice and examined one month later (as done before B). All data were obtained by using a 1/50 dilution. ☐ plasma, ■ induced ascites.

When other mice were injected with these tumours, higher levels of anti-CRP were not induced, even though large amounts of cells and ascites were produced. Activity ranged from 0.7 and 0.3 OD 450 of plasma and ascites respectively in the high producers, to 0.3 and 0.15 OD 450 in the low producers. Nine hybrids were selected from those with the most activity *in vivo*, good growth *in vitro*, and the smallest number of estimated different clones. These were the mice designated with the numbers 1, 2, 3, 5, 6, 9, 13, 15 and 16.

The nine hybrids were thawed, then further grown *in vitro* and injected into pristane primed mice. They were given 3-5 injections in dosages of 1-5 × 10^6 cells/mouse. Control mice received injections of culture medium only. One month later both plasma and ascites were examined for anti-CRP activity. Figure 3B shows that even though all the clones selected had higher levels of activity in plasma (most values were between 0.8 and 1.4 OD 450 units), only clones 1 and 5 had high levels of induced ascites (0.7 and 1.4 respectively). Cells from mouse number 1 were estimated to be monoclonal and cells from number 5 were estimated to have a maximum of 3 clones.

Further growth of clones 1 and 5 allowed for the amplification and production of ascitic fluid, with large amounts of anti-CRP activity. Figure 4 shows titre estimation of anti-CRP activity obtained from the induced ascitic fluid, and then compared with polyclonal mouse anti-CRP. At 1/500 dilution, the 5 ml induced ascitic fluid clone 5 still had 0.5 OD 450 units, whereas the polyclonal ascites needed less than 1/50 dilution to show this same activity. The mice could be injected and drained every other day without titre loss for approximately 2-3 weeks.

Specificity. The solid phase assay was used to investigate the binding

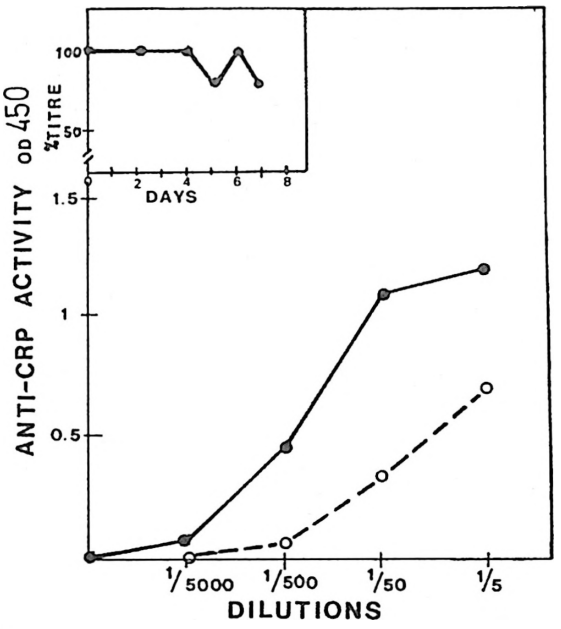

Fig. 4. *Ascitic fluid titre induced in a mouse injected with clone 1.*

A mouse received intraperitoneally about 5 × 10^6 cells, distributed in 5 injections during a one month period. Five days after the last injection, it was injected intraperitoneally with 5 ml of 0.8 % NaCl and allowed to stand for 30 minutes. After this time it was drained and the 5 ml recovered were centrifuged. Anti-CRP activity measurements were done by ELISA as described beforehand. Draining was repeated for several days and titres were measured by the same procedure (upper right corner). (•) D5, (o) polyclonal anti-CRP produced by CRP injection into mice and subsequent ascite induction with parenteral myeloma.

specificity of the monoclonal antibodies obtained from the ascitic fluids. Figure 5 shows the competition of monoclonal anti-CRP 1 with CRP. About 0.6 OD 450 units could be reduced to 0.2 by incubating the ascites simultaneously in the ELISA well (0.25 μg of bound CRP) with 5 μg of pure CRP. This inhibition could be reversed by using proteinase K treated CRP instead. Similar results were obtained with other anti-CRP monoclonals.

The ascites were purified by prot A-linked agarose chromatography (11). The elution profiles of both hybrid induced ascitic fluid and control ascites were measured through anti-CRP activity. The pooled peaks from the hybrid ascites resulted in positive ELISA. However, the pooled peaks from the control ascites showed no activity (data not shown).

To further assure the specificity of the anti-CRP activity contained in the ascitic fluid, it was allowed to react for 1 h at 37° C with pure CRP (300 μl of ascites, 50 μg CRP and 2.5 mM $CaCl_2$).

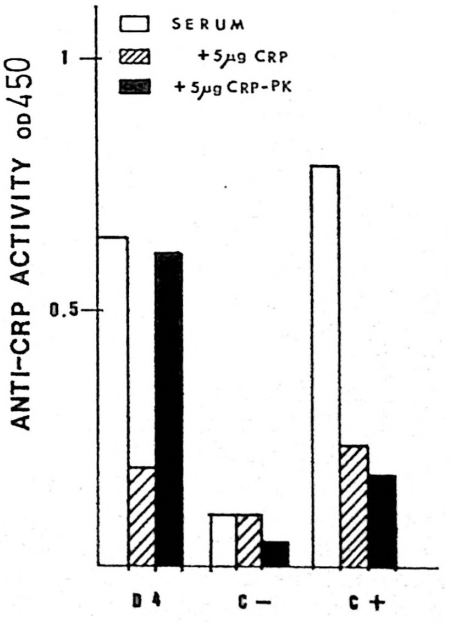

Fig. 5. *Competition of monoclonal anti-CRP with CRP.*

The experiment was performed in 96 well polyvinyl plates covered with 0.25 μg CRP/well. Ascites were diluted 1/50 with culture medium. Other details were as described in *Materials and Methods*. During the incubation of plates and ascites, some wells received 5 μg of CRP treated with proteinase K (2.5 mg/ml) at 37° C overnight. The (C—) indicates normal induced ascites and the (C+) the polyclonal anti-CRP produced by an injection of CRP into mice, and subsequent ascite induction with parental myeloma.

Then Sigma anti mouse IgG (30 μl 1/100 dilution) was added and incubated overnight at 4° C. The precipitate was centrifuged and washed with 10 mM Tris pH 6.8. The presence of CRP in the precipitate was detected by both SDS gel electrophoresis stained with silver nitrate, and by ELISA-sandwich (*Materials and Methods*).

Discussion

Recent interest in the quantification of human CRP responds to the potential value of this protein as a nonspecific tumour-development marker, and in the differential diagnosis between certain diseases (8, 12). CRP levels cannot be measured automatically, precisely and reproducibly by the current methods based on conventional antibodies. Thus, monoclonal antibodies were developed to provide these measurements. Since one of the technical problems in the large scale production of monoclonal antibodies is the stability of the hybrids (11), a method was devised to select them.

The method described in this paper has thus far demonstrated how two high stability hybrids can grow *in vitro* or *in vivo* and produce high titre monoclonal antibodies (figure 3). The strategy used consisted in allowing the hybrids to grow for about a month, and recover from freezing-thawing cycles, before cloning. Whereas most procedures (11) performed cloning immediately after fusion, this method grows the hybrids long enough (fig. 1) before cloning, in order to select a producer that competes with the most vigorous non-producer hybrids present in the mixture after fusion. Furthermore their survival throughout freezing was assured before attempting any cloning procedure. Cloning was otherwise as described by most methods (4, 6). In this manner, 18 clones were selected from one fusion for further study (fig. 2).

After cloning growth in the mice was brought about in increasing cell concentrations. Nine clones were selected, having high serum titres with injections of vey small quantities of cells, good *in vitro* growth and few clones. Mice injected with these at higher cell concentrations, allowed for the selection of 2 high titre producers (fig. 3). Large scale production has been demonstrated (figure 4). Recloning was done to reassure the stability of the isolated clones.

Preliminary characterization of both antibodies included mainly: competition with CRP, binding to protein A sepharose, stability studies and antigen recovery from antigen-antibody complexes by both ELISA and SDS gel electrophoresis (fig. 5). The instability of monoclonal antibodies against CRP has also been described, especially among those belonging to IgM (7).

The antibodies described in this paper provide both high activity and high stability probes to be used in CRP quantification on a clinical escale. At this time, conventional antibodies in a sandwich ELISA are being substituted by monoclonal antibodies.

Acknowledgements

We would like to express our appreciation for their help to the following persons, acknowledgeing their participation in the presentation of this manuscript: M. Zamora: gel electrophoresis experiments; S. Martín: CRP quantification by ELISA sandwich; J. Coll-Pérez: drawings (illustrations); R. Olaizola-Garbisu: typing; A. Steele: revision of English text.

M. Iturralde was provided with a grant from the «Caja de Ahorros de Zaragoza, Aragón y Rioja», as financial aid in carrying out this investigation.

Resumen

Se expone la obtención y selección de híbridos con alta estabilidad, productores de anticuerpos

monoclonales contra la proteína C reactiva humana.

Esta nueva estrategia consiste en desarrollar los híbridos y congelarlos antes de clonarlos para asegurar su estabilidad y seleccionar los híbridos de aquellos que producen altos títulos en el líquido ascítico inducido del ratón.

Se han obtenido de este modo, dos anticuerpos monoclonales con alta estabilidad y gran potencial para su producción a gran escala.

La producción a gran escala de los anticuerpos monoclonales contra la proteína C reactiva humana puede usarse en la cuantificación clínica y para estudios fisiológicos sobre su función *in-vivo* que aún permanece desconocida.

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