# T-cell lines producing antigen-specific suppressor factor

THE immune system is now recognised as a network of interacting cells with some interactions augmenting, and others suppressing immune responses. Regulatory interactions are often mediated by T cells, and do not always require cell-to-cell contact between appropriate cells, but may be mediated by soluble transmitters, commonly termed 'factors'. Both antigen-specific[1-7] and nonspecific[8-10] factors have been described. Biochemical characterisation of these factors is hampered by the often minute quantities required to mediate biological effects and by the multiplicity of regulatory pathways involved in immune responses. Our knowledge of these factors is derived from functional tests, often combined with serological analysis of the factors. One approach to the analysis of monoclonal cell products is the somatic cell hybridisation technique described by Milstein *et al.*[11,12]. Using this method, specific antibody-forming cells have been hybridised with myeloma cells, and have yielded clones producing specific antibody[11]. This antibody is particularly useful as it is monoclonal and large quantities can be obtained. Although attempts to produce T-cell hybrids have been successful[13-15], the products have not been functional. However, only failures in expression of cytotoxic activity have been described[13-15]. We describe here one antigen-specific suppressor factor (SF)-producing line S1-41 and its products.

**Table 1** Specificity of suppression and molecular characterisation of S1-41 hybridoma factor SF S1-41

| HC | Stimulus antigen | Suppression SF (%) | Response (anti-DNA AFC per culture) | % Suppression |
|---|---|---|---|---|
| Expt *A*— | TNP–KLH | — | 33±10* | — |
| $HC_{KLH}$ | TNP–KLH | — | 820±92 | 0 |
| $HC_{KLH}$ | TNP–KLH | CBA $SF_{KLH}$ 10 | 313±19† | 64 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 10 | 133±39† | 87 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 10 | 313±24† | 64 |
| Expt *B*— | DNP–GAT | — | 73±12† | — |
| $HC_{GAT}$ | DNP–GAT | — | 383±7 | 0 |
| $HC_{GAT}$ | DNP–GAT | SF S1.41 10 | 613±56 | 0 |
| $HC_{GAT}$ | DNP–GAT | SF S1.41 1 | 493±26 | 0 |
| Expt *C*— | TNP–PAA | — | 3,370±82 | 0 |
| — | TNP–PAA | SF S1.41 10 | 3,550±262 | 0 |
| — | TNP–PAA | SF S1.41 1 | 3,743±471 | 0 |
| Expt *D*— | TNP–KLH | — | 90±43† | — |
| $HC_{KLH}$ | TNP–KLH | — | 1,400±178 | 0 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 | 333±31† | 81 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 abs. KLH | 1,433±129 | 0 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 elu. KLH | 313±29† | 83 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 abs. GAT | 260±85† | 87 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 elu. GAT | 1,293±24 | 8 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 abs. $\alpha Ia^k$ | 1,613±84 | 0 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 elu. $\alpha Ia^k$ | 593±81‡ | 62 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 abs. $\alpha Ia^s$ | 493±64† | 71 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 elu. $\alpha Ia^s$ | 1,513±182 | 0 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 abs. $\alpha I\text{–}J^k$ | 1,480±111 | 0 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 elu. $\alpha I\text{–}J^k$ | 307±73† | 83 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 abs. $\alpha I\text{–}J^s$ | 240±84† | 89 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 elu. $\alpha I\text{–}J^s$ | 1,267±131 | 10 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 abs. αMig | 300±51† | 84 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 elu. αMig | 1,370±278 | 2 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 +RαSF | 1,520±96 | 0 |
| $HC_{KLH}$ | TNP–KLH | SF S1.41 +MαSF | 1,453±152 | 0 |

For hybridisation, $10^8$ *in vitro* induced KLH-specific suppressor cells of CBA origin[19], and $10^7$ BW5147 cells (a HGPRT-negative AKR thymoma line obtained from R. Hyman) were washed twice in serum-free balanced salt solution (BSS) and pelleted together at 400*g*. Polyethyleneglycol (0.5 ml; PEG, BDH, molecular weight 1,500) 50% in BSS, *p*H 7.8, was added drop by drop over 2 min as the cells were gently shaken into suspension. Serum-free BSS (0.5 ml) was added at the same rate and then drop by drop a further 5 ml BSS before slowly filling the tube to 20 ml with BSS. The cells were spun at 400*g*, the supernatant discarded and the cells resuspended in 100 ml Dulbecco's minimum essential medium (DMEM; Flow) with 20% fetal calf serum (FCS) and dispersed in 2-ml aliquots into 48 wells of Linbro trays (Flow). At 24 h after the fusion, 1.0 ml DMEM plus 20% FCS was removed and replaced by 1.0 ml HAT medium (DMEM plus 20% FCS containing $1.1\times10^{-4}$M hypoxanthine, $1.6\times10^{-5}$M thymidine and $4\times10^{-7}$M aminopterin) and this procedure was repeated on the next 2 d. On days 6, 8 and 10, HT medium was used (DMEM plus 20% FCS containing $1.1\times10^{-4}$M hypoxanthine and $1.6\times10^{-5}$M thymidine). Thereafter, (day 13 after fusion) the medium was changed to DMEM plus 10% FCS, and the contents of each Linbro well which started to grow within the next 1–2 weeks were subcultured in Linbro wells and then transferred to Nunc flasks (50 ml, Nunclon-Delta 1461). Aliquots of supernatants were tested for function when the cells were growing in the Linbro plates, and subsequently in the flasks. For the assay, Marbrook–Diener cultures were incubated in a humidified atmosphere of 10% $CO_2$ in air at 37°C (ref. 20). Hybridoma supernatants were added to the mixture of KLH-, GAT- (copolymer of L-glutamine$^{60}$-L-alanine$^{30}$-L-tyrosine$^{10}$- or (T,G)–A--L (poly (L-tyrosine-L-glutamine)–poly(DL-alanine)–poly(L-lysine)- primed helper cells ($HC_{KLH}$, $HC_{GAT}$ or $HC_{(T,G)\text{-}A\text{-}L}$ (primed *in vitro* as described previously[19]), normal spleen cells, and haptenated antigens TNP–KLH, DNP–GAT or DNP-(T,G)–A--L (ref. 18). The anti-DNP antibody-forming cells (AFC) were assayed on day 4 of the *in vitro* cooperative culture[18]. With unprimed B cells only IgM anti-DNP AFC were detected. All the cultures were carried out in triplicates and assayed separately. The assay is summarised as:

Spleen cells + haptenated antigen = background
Helper cells + spleen cells + haptenated antigen = help
Helper cells + spleen cells + haptenated antigen + suppressor cells S/N(SF) = suppression

Results are expressed as numbers of anti-DNP AFC per culture ± s.e. The degree of suppression was calculated as follows:

$$\%\ \text{Suppression} = 100 - ([\text{suppression} - \text{background}/\text{help} - \text{background}] \times 100)$$

KLH-specific suppressor cells were induced by 4 d *in vitro* culture with 100 μg KLH $ml^{-1}$ (ref. 17), and fused with BW5147 cells on day 4 of culture. The medium used in the induction of helper or suppressor cells and in cooperative cultures was Eagle's minimum essential medium (MEM Gibco, with added bicarbonate 3.7 g $l^{-1}$ and 5% FCS) in the outside medium, and HEPES-buffered (4.36 g $l^{-1}$) MEM with 5% FCS in the inserts. *Expt A*, $3\times10^5$ BlO.BR $HC_{KLH}$ were added to $10^7$ normal BlO.BR spleen cells and the mixture was stimulated with 0.1 μg TNP–KLH $ml^{-1}$, SF were added to appropriate groups at the beginning of the 4-d cooperative culture. IgM anti-DNP AFC were assayed on day 4 of culture. The effect of SF S1.41 on KLH-specific help has been tested about 25 times over a period of 14 months. *Expt B*, lack of effect of SF S1.41 on helper cells of another specificity is shown by cultures containing $10^5$ BlO.BR $HC_{GAT}$, $10^7$ normal BlO.BR spleen cells and 1.0 μg DNP–GAT $ml^{-1}$. IgM anti-DNP AFC were assayed as in expt *A*. The specificity of SF S1.41 suppression has been tested on GAT or (T,G)–A--L-specific help four times over a period of 14 months. *Expt C*, lack of effect of SF S1.41 on a thymus-independent response is shown. $10^7$ normal BlO.BR spleen cells were stimulated with TNP–PAA (polyacrylamide beads) 0.3% final concentration in the absence or presence of SF S1.41. The assay of anti-DNP AFC was as in expt *A*. Two other experiments gave similar results. *Expt D*, $8\times10^5$ BlO.BR $HC_{KLH}$ were added to $10^7$ normal CBA spleen cells, and the mixture was stimulated with 0.1 μg TNP–KLH $ml^{-1}$ in the presence or absence of SF S1.41. The assay of anti-DNP AFC was as in expt *A*. For molecular characterisation of S-41 supernatant SF S1.41, the supernatant of S1.41 was absorbed and 'acid eluted' (using Sörensen's buffer *p*H 2.4) from the following immunoabsorbents: KLH (1 mg KLH/ml beads), GAT (1 mg GAT/ml beads), $\alpha Ia^k$ ($\alpha Ia^k$ serum was ATH anti-ATL 0.5 ml/5 ml beads), $\alpha I\text{–}J^k$ (BlO.HTT anti-BlO.S(9R) or BlO.A(3R) anti-BlO.A(5R) serum, 0.5 ml/5 ml beads), $\alpha\text{–}J^s$ (BlO.S(9R) anti-BlO.HTT serum, 0.5 ml/5 ml beads), and αMIG (0.5 ml rabbit anti-mouse Ig/5 ml beads). The beads used were Sepharose 4B (Pharmacia). The cyanogen bromide activation of beads and coupling of antigen or antisera was as described[22]. Preparation of anti-SF antisera and their characteristics are documented elsewhere[17]. They were used in cultures at 0.1% final concentration. Two other experiements of type D yielded similar results. Within each experiment the numbers of AFC in each group were compared to that of the positive controls (helper cells, normal spleen cells and antigen, second line in each experiment) using Student's *t*-test. *P* values are marked in the Table as follows: $*P \leqslant 0.001$; $\dagger P \leqslant 0.01$; $\ddagger P \leqslant 0.05$.

Because of our interest in antigen-specific soluble mediators of T-cell function, we decided to hybridise a thymoma line with *in vitro* induced antigen-specific suppressor T cells, known to release antigen-specific SF into the medium[6]. SF has been shown by the use of immunoadsorbents to have a combining site for antigen, Ia (I-J) coded determinants, no conventional Ig, but determinants reacting with anti-'constant' region and anti-'variable' region anti-SF antisera[16,17] in the same molecule. SF acts on T helper cells of the same antigenic specificity, and shows no genetic restrictions in its function[18]. Two hybridisation experiments of this kind have been carried out so far and eight suppressor cell lines established. In the present experiment with cell line S1-41, a nonspecific suppressor cell line S1-34 and its product SF S1-34 was used as a control. Cell hybridisations were carried out essentially as described previously[11–13], using polyethyleneglycol.

In the first hybridisation 20% of the supernatants from Linbro wells containing growing cells showed suppressive activity. The majority of these suppressor hybridomas were later found to be contaminated with bacteria, but the remaining two, S1-41 and S1-34, were both of interest, one showing antigen-specific suppression (S1-41), the other nonspecific suppression (S1-34). S1-41 has now been cloned but the present report is of data obtained using supernatants from the uncloned line (SF S1-41). In the second hybridisation 20% of the hybridomas were initially selected as being suppressive; from these, six lines were established, some showing specific suppression and others nonspecific suppression. As shown in Table 1, SF S1-41 suppressed primary *in vitro* responses to the same extent as conventional suppressor factor CBA $SF_{KLH}$ obtained from suppressor cells. This suppression is antigen specific, and does not affect help for responses of other antigenic specificity as shown on lines 6–9 of Table 1. Thymus-independent responses are also not affected, as shown by lines 10–12 of Table 1. The activity of SF S1-41 is absorbed out by the specific antigen (KLH), $\alpha Ia^k$ and $\alpha I\text{-}J^k$, but not by an irrelevant non-cross-reactive antigen (GAT), $\alpha Ia^s$, $\alpha I\text{-}J^s$, or by rabbit antimouse immunoglobulin ($\alpha$MIG) coupled to solid-phase immunoabsorbents, as shown in experiment D in Table 1. These results suggest that SF S1-41 carries an antigen-combining site and Ia(I-J)-coded determinants in the same molecule. Furthermore, the activity of SF S1-41 is abolished by rabbit or mouse (syngeneic) antisera to CBA $SF_{KLH}$ (last two lines of experiment D, Table 1). Rabbit anti-SF (R$\alpha$SF) inactivates all SF regardless of their strain of origin or specificity, and thus recognises 'constant' region-like determinants[17]. CBA anti-CBA$SF_{KLH}$(M$\alpha$SF) only inhibits the activity of CBA $SF_{KLH}$ and thus recognises 'idiotype-like' determinants of the combining site of SF[17]. These results show that SF S1-41 carries both the 'constant' and 'variable' region determinants of conventional suppressor factors; thus, SF S1-41 resembles conventional SF in all known aspects tested, including a molecular weight about 50,000–60,000 (refs 16, 17).

The effect of SF S1-41 on a secondary response is shown in Table 2, in which comparable suppression is obtained with a conventional SF and SF S1-41 on both IgM and IgG responses. For comparison, the effect of addition of SF S1-34 is shown. SF S1-34 is not antigen specific, and the molecule does not carry 'constant' region or 'idiotype-like' determinants (data not shown). The suppression by SF S1-41 and SF S1-34 has been tested repeatedly over a period of 14 months, with no change in suppressive capacity.

The Thy-1.2 (CBA parent) and Thy-1.1 (BW 5147) surface membrane phenotypes of the hybridomas were tested by direct cytotoxicity 1, 9 and 12 months after hybridisation. Ly-1, Ly-2 and Ly-6 phenotypes were tested by absorption 12–14 months after hybridisation. An example of data from the absorption analysis is shown in Fig. 1. The full phenotypes are as follows: S1.41, Thy-1.1$^+$ Thy-1.2$^+$ Ly-1.1$^+$ Ly-1.2$^+$ Ly-2.1$^-$ Ly-6.2$^+$, S1-34, Thy-1.1$^+$ Thy-1.2$^+$ Ly-1.1$^-$ Ly-1.2$^-$ Ly-2.1$^-$ Ly-6.2$^+$, BW, Thy-1.1$^+$ Thy-1.2$^-$ Ly-1.1$^-$ Ly-1.2$^-$ Ly-2.1$^-$ Ly-6.2$^+$. The Thy-1 phenotypes at least have been stable over 14 months.

Our results suggest that T-cell hybrids can be produced fairly readily when *in vitro* primed T cells, either T suppressor or T helper cells (unpublished) are used as a T-cell source. In the light of reported failures in obtaining functional T-cell hybrids[13–15] the high frequency at which functional hybrids were obtained (20% of hybridomas being functional) may be surprising. It may be that T cells which have been recently exposed to antigen hybridise more readily, and after priming *in vitro* may adapt to further *in vitro* culture more easily.

The phenotypes of the hybrids are intriguing. S1-41 expresses both parental Thy-1 types (data not shown) confirming that it is a hybrid between AKR and CBA cells. Both parental Ly-1 alleles are also expressed. This is particularly interesting as our subline of BW5147 does not express Ly-1.2 (see Fig. 1). This suggests that although BW5147 has the structural gene for Ly-1.2, it has lost a gene controlling expression of the antigen. Presumably, this is provided by the CBA parent in the S1-41 hybrid. On the other hand, S1-34 fails to express either Ly-1 or Ly-2 of either parent.

Neither the phenotype of S1-41 nor S1-34 correspond to the phenotypes previously described for specific or nonspecific suppressors—respectively Ly-1$^-$2$^+$ and Ly-1$^+$2$^+$ (refs 23–25). It therefore seems that there is a dissociation between expression of Ly surface components and function in these two hybridomas. Alternatively, S1-41 may have some of the properties of suppressor cell inducers which interact with suppressor cell precursors, and are Ly 1$^+$ (refs 25, 26).

**Table 2** Effect of suppressor cell hybridoma S1-41 supernatant SF S1-41 on secondary antibody responses

| Stimulus | | Suppression | | Response (Anti-DNP AFC per culture ± s.e.) | | | |
|---|---|---|---|---|---|---|---|
| Spleen cells | Antigen | SF | Conc.(%) | IgM | (%S) | IgG | (%S) |
| CBA TNP–KLH 1° | — | — | — | 67 ± 37* | (—) | 87 ± 37* | (—) |
| CBA TNP–KLH 1° | TNP–KLH | — | — | 2,300 ± 147 | (0) | 8,425 ± 485 | (0) |
| CBA TNP–KLH 1° | TNP–KLH | CBA $SF_{KLH}$ | 10 | 1,617 ± 160† | (40) | 5,067 ± 386† | (40) |
| CBA TNP–KLH 1° | TNP–KLH | CBA $SF_{KLH}$ | 1 | 1,000 ± 157† | (58) | 7,050 ± 560 | (16) |
| CBA TNP–KLH 1° | TNP–KLH | CBA $SF_{KLH}$ | 0.1 | 1,183 ± 177† | (50) | 7,318 ± 1049 | (13) |
| CBA TNP–KLH 1° | TNP–KLH | SF S1-41 | 10 | 667 ± 100* | (73) | 4,600 ± 83† | (46) |
| CBA TNP–KLH 1° | TNP–KLH | SF S1-41 | 1 | 950 ± 72† | (60) | 6,683 ± 463 | (30) |
| CBA TNP–KLH 1° | TNP–KLH | SF S1–41 | 0.1 | 1,483 ± 108‡ | (37) | 6,050 ± 707‡ | (28) |
| CBA TNP–KLH 1° | TNP–KLH | SF S1-41 | 0.01 | 1,867 ± 254 | (19) | 3,867 ± 132* | (55) |
| CBA TNP–KLH 1° | TNP–KLH | SF S1-34 | 10 | 1,633 ± 201† | (30) | 4,233 ± 372‡ | (50) |
| CBA TNP–KLH 1° | TNP–KLH | SF S1-34 | 1 | 1,167 ± 174‡ | (57) | 4,117 ± 263* | (52) |
| CBA TNP–KLH 1° | TNP–KLH | SF S1-34 | 0.1 | 1,667 ± 204 | (28) | 4,195 ± 1095† | (51) |

CBA mice primed with TNP–KLH on bentonite were boosted with 20 μg TNP–KLH in saline intravenously 7 d before the cultures were started. $3 \times 10^6$ primed spleen cells were cultured in the presence or absence of SF. The stimulation was by 0.01 μg ml$^{-1}$ TNP–KLH per culture[16]. IgM and IgC (developed by goat anti-IgM and sheep anti-IgG[16]) anti-DNP AFC were assayed on day 6 of culture. Percentage suppression (%S) is given in parenthesis. Three other experiments gave similar resulrs. *P* values are marked as in Table 1.

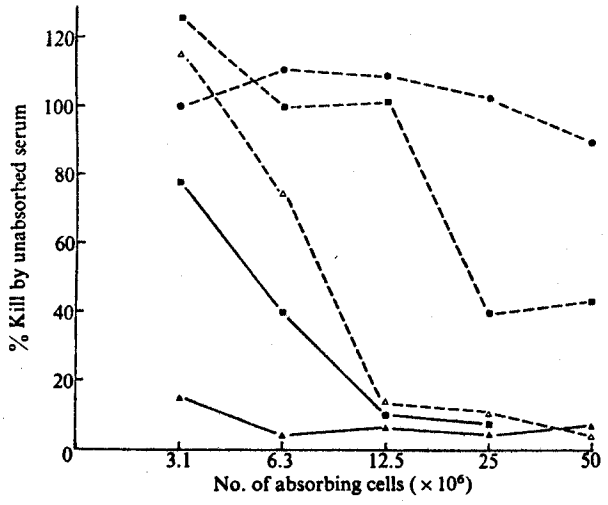


**Fig. 1** Absorption analysis of the Ly surface membrane phenotype of hybrids. Preparation, testing and if necessary absorption of Ly sera and rabbit complement was as described previously[23]. Sera were absorbed at the last dilution giving plateau lysis of appropriate thymocyte targets. Aliquots (50 μl) of anti-Ly-1.1 (——) anti-Ly-1.2 (– – –), or anti-Ly-2.1 (data not shown) were absorbed for 1 h at room temperature with $3–50 \times 10^6$ tissue culture grown BW5147 (●), S1.41 (■), S1.34 (data not shown), or freshly prepared CBA (▲) or B10 thymocytes (△). Absorbed sera were assayed by a microcytotoxic test on CBA or B10 thymocytes. 2 μl antiserum was added to 2 μl cell suspension (2,000 cells in Hank's BSS+5% FCS) in the wells of a microtitre plate (Falcon microtest 3034), and incubated at 37°C for 15 min. One drop of medium was then added and the plate refrigerated for 10 min. The supernatant was then removed by inverting and flicking the plate; 2 μl 1/12 rabbit complement was added and the plate incubated at 37°C for 30 min. A drop of Trypan blue was then added, the plate again refrigerated and flicked, and a further drop of medium added for counting. For each antiserum, positive and negative control absorptions with appropriate thymocytes were carried out in each experiment. For simplicity, the negative control absorptions and negative results obtained with BW5147, S1.41 and S1.34 have been omitted from the figure, except for the example of anti-Ly-1.2 absorbed with BW5147. Other negative results showed essentially the same pattern.

The secreted suppressor factor of the S1-41 hybridoma, SF S1-41 showed no change in function or specificity over a period of 14 months. SF S1-41 has retained all the characteristics of conventional antigen-specific SF, carrying in the same molecule, antigen combining site, Ia (I-J) coded determinants and 'constant' region and 'idiotype-like' determinants of CBA $SF_{KLH}$. The latter are defined respectively by rabbit and mouse antisera raised against mouse SF[17], and the determinants recognised by the sera have been termed 'constant' and 'idiotypic' or 'variable' by analogy with those parts of conventional Ig. It should be emphasised, however, that there is no evidence for the presence of conventional Ig determinants on either CBA $SF_{KLH}$ (ref 16) or SF S1-41. Furthermore, S1-41 cells bear only T-cell markers Thy-1, Ly-1 and Ly-6, and do not carry surface Ig (unpublished). The characteristics of other specific and non-specific suppressor hybridomas and their products are under study as is the *in vivo* effect of the S1-41 hybridoma.

Our results show that functional T-cell hybrids can be produced by standard techniques and that the established hybrids are stable as judged by surface membrane phenotype and the functional properties of their secreted products. Such hybrids will be extremely useful in the more refined characterisation of the secreted products of T cells of various types, as unlimited cell numbers can be produced and the titre of SF produced by the hybrids is much higher than that of conventional factors.

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# Most IgM-producing cells in the mouse secrete auto-antibodies (rheumatoid factor)

IN the mouse the injection of bacterial lipopolysaccharide (LPS) results in the appearance of very large numbers of IgM globulin-secreting cells[1]. Furthermore, it has been shown that LPS induces B lymphocytes, both *in vivo*[2,3] and *in vitro*[4–6] to secrete IgM antibodies to a wide range of seemingly unrelated antigens. It was reported recently that about 8% of all IgM globulin-secreting cells arising as a consequence of LPS injection into CBA mice secrete an antibody-like molecule with specificity for a heterospecific (bovine) IgG (BIG) molecule[1]. It was suggested that such IgM molecules were reminiscent of a rheumatoid factor, although at that time

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IMMUNOGLOBULIN PRODUCING HYBRID **Hybrid Cell Lines**

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## 17.1 INTRODUCTION

The problems of producing and characterizing antisera were radically altered when Köhler and Milstein (1975) demonstrated that antibody-producing hybrid cell lines could be generated by somatic cell hybridization. Within three years, antibodies of defined specificity produced by continuous cultures of monoclonal cell lines became routine laboratory reagents providing exquisite serological and biochemical probes (Oi et al. 1978). This chapter describes a modified version of the technique described by Galfre et al. (1977) to fuse murine spleen cells with the 8-azaguanine-resistant, nonsecreting mouse myeloma cell line NS-1 (a derivative of MOPC-21 (P3)). In this procedure polyethylene glycol is used as the fusion agent (Pontecorvo 1976) and Littlefield's hypoxanthine-aminopterin-thymidine (HAT) medium (Littlefield 1964) is used for selecting stable, monoclonal antibody-producing hybrid cell lines.

From the start (immunizing mice and growing the myeloma cell parent) to the end (when stable, cloned cell lines have become established), this method requires three to four months of continuous bench work. In addition, the generation of monoclonal antibody-producing cell lines demands constant attention and should not be undertaken unless one can afford both the time and the effort. Moreover, it is necessary to establish the antibody detection system to analyze the products of the hybrid cell lines before beginning hybridization because there may not be sufficient time to work out technical problems after hybrid cells begin to grow.

Tissue culture facilities for generating antibody-producing hybrid cell lines minimally require the following equipment and supplies:

1. Tissue culture hood
2. Humidified incubator with a $CO_2$-in-air atmosphere
3. Bench top centrifuge
4. Inverted phase contrast microscope
5. Bright field microscope
6. Tissue culture supplies
7. Liquid nitrogen storage containers

In addition, investigators using these methods will require the laboratory equipment necessary for producing antibody in usable amounts, analyzing antibody activity, and characterizing the antibody molecules produced by the hybrids. The exact equipment and materials required for each of these operations will depend on the nature of the antibodies being produced and the scale to which production is carried out.

The procedure we describe for the development and use of antibody-producing hybrid cell lines (Figure 17.1) includes growing the parental myeloma cell line, immunizing mice to provide immune donor spleen cells, conducting the cell fusion, and selecting the resulting hybrids with HAT medium. Supernates of the surviving hybrid cell cultures are then tested for antibody activity. An aliquot of cells from the antibody-producing cultures is grown and prepared for freezing while another aliquot is employed in cloning the hybrids. To derive cloned cell lines, the cells are grown at limiting dilutions. Clones that secrete the desired antibody are expanded, and several aliquots of these clones are frozen while others are used for the large-scale production of antibody. The resulting antibodies are purified and characterized.

## 17.2 PREPARATION OF THE FUSION PARTNERS

### A. NS-1 Myeloma Parent Cell Line*

NS-1, abbreviated from P3-NS1-1, is a cell line derived from MOPC-21, a BALB/c myeloma cell line. NS-1 does not produce the MOPC-21 $\gamma_1$ heavy chain; it synthesizes the original MOPC-21 $\kappa$ chain but internally degrades it. This line is 8-azaguanine resistant and therefore susceptible to HAT selection (see Section 17.4). The advantage in using NS-1 as the fusion myeloma parent cell line, rather than other myeloma cell lines (such as P3-X63Ag8), is its inability to produce a heavy chain. Antibody-producing hybrid cell lines derived with this fusion partner will produce mixed molecules with only the MOPC-21 $\kappa$ light chain; the molecules will not have both light and heavy chains derived from the myeloma cell (see Section 17.10).

* Three new cell lines have been developed for somatic cell hybridization with immune spleen cells to generate antibody-producing hybrid cell lines. The two mouse lines Sp2/0-Ag14 (Shulman et al. 1978) and P3X63-Ag8.653 (Kearney et al., submitted for publication) are total nonsecretors. They do not synthesize either light or heavy chains, and hybrid cells derived with these parental cell lines will only produce antibody of the spleen cell parent. The third cell line is a rat myeloma cell line, Y3-Ag1.2.3 (Galfre et al. 1979), which secretes light $\kappa$ chains. Antibody-producing hybrid cell lines are derived from rat spleen cells fused with a mouse myeloma cell line are difficult to grow as tumors in either mice or rats. The development of this myeloma cell line permits the production of hybridomas using rat cells for both fusion partners. The resulting hybrids can be adoptively transferred for growth and antibody production in rats because these cells present no xenogeneic antigens to the host.

Not all NS-1 cell lines are equivalent. Some sublines of NS-1 have been reported not to give rise to antibody-producing hybrid cell lines. Therefore, care should be taken when recloning NS-1. Recloning is necessary to maintain a homogeneous cell line; long-term maintenance of NS-1 (or any other cell line) in tissue culture gives rise to spontaneous genetic drift of the cell population. Such drift affects many characteristics, for example, continued resistance to 8-azaguanine. Upon recloning NS-1, testing of the new NS-1 clone for the ability to fuse with immune spleen cells and yield antibody-producing hybrid cell lines is recommended. This is a tedious procedure. If a particular cell line is a good fusion partner, establishing frozen stocks of the cells as a continual source of the original cell line is the best way of assuring continued success in generating antibody-producing hybrid cell lines.

#### 1. Maintaining NS-1

##### MATERIALS AND REAGENTS

NS-1 myeloma cell line (P3-NS1-1; Cell Distribution Center, Salk Institute)
Culture medium:
- RPMI 1640
- L-glutamine
- Sodium pyruvate
- Penicillin
- Streptomycin
- Fetal calf serum (FCS), screened (see comment 1)

$CO_2$ incubator, humidified
7% $CO_2$-in-air gas mixture
Stationary T flasks, roller bottles, or Spinner flasks

## PROCEDURE

1. Culture NS-1 cells in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin, and 15% FCS in a humidified 7% $CO_2$ in air atmosphere at 37°C.
2. NS-1 can be grown either in stationary T flasks, roller bottles, or Spinner flasks. In any of these containers, NS-1 and hybrid cell lines derived from NS-1 grow to a maximum density of 2–5 × $10^5$ cells/ml with doubling time of 12–24 hours. When this density is reached, there is a precipitous fall in cell viability. Generally NS-1 cultures must be split (i.e., diluted either by removing the contents of the old flask and adding fresh medium or by addition to a new flask with medium) every 3–4 days. A 1-in-10 or 1-in-20 split, depending on the condition of the cells, is recommended. Cultures with cell densities lower than $10^4$ cells/ml grow poorly.

## COMMENTS

1. The FCS should be a screened lot known to support the clonal growth of at least the parental myeloma cell line NS-1. To this capacity, clone NS-1 with batches of FCS by limiting dilution (see Section 17.7) without feeder cells. A good serum lot will support 100% cloning efficiencies of NS-1, but sera yielding 70–80% cloning efficiencies are satisfactory. Generally, 10–20% of the serum lots tested are suitable. Most commercial suppliers of FCS will reserve serum lots for three weeks while one is determining whether the lot is usable. Obviously, only serum lots with adequate quantities in reserve should be tested.
2. The 7% $CO_2$-in-air atmosphere assures a slightly acidic medium pH. NS-1 and hybrid cell lines derived from NS-1 grow better under such conditions. These cells are somewhat intolerant of basic pH medium. (Adequate buffering of RPMI 1640 medium is provided by 5–10% $CO_2$-in-air atmospheres.)
3. Should one find that a NS-1 cell culture has overgrown and is dying, it is better to start again from frozen stock than to attempt to salvage the culture. Selective forces in the dying cultures might result in the growth of cells with altered phenotypes that make them unsuitable fusion partners.

### 2. Preparation of NS-1 Fusion

A total of 1.5 × $10^8$ NS-1 cells is generally used for fusion. These cells should be in log-phase growth. To insure that the cells are in log-phase growth, they should be at a density of about $10^5$ cells/ml. Thus, 1–2 liters of NS-1 cells are grown in preparation for fusion. Cell viability at the time of collecting the cells should be greater than 95%. Because cell viability determinations with trypan blue are influenced by the presence of serum albumin, either the albumin must be washed away before cell counts are made or another method of determining viabilities must be used. A convenient method using acridine orange-and-ethidium bromide and fluorescence microscopy is described in Section 1.15.

### B. Immune Spleen Cells

Mice are generally immunized with limited immunization protocols to provide the immune spleen cells used for fusion. In most cases, hyperimmunization is not necessary.

1. If the antigen is soluble, prime mice intraperitoneally with 100 $\mu$g of antigen precipitated in alum, mixed with $2 \times 10^9$ killed *Bordetella pertussis* organisms. (See Section 2.5 for sources of reagents and procedures for alum precipitation of antigen.) Boost mice (intraperitoneally or intravenously) 1–3 weeks later with 10$\mu$g aqueous antigen without *B. pertussis*. Use the spleen cells for fusion 3 days after the boost. Best results have been obtained using this immunization protocol. Alternatively, fusion can be done 3–4 days after the priming dose, or 3 days after a second boost.
2. If the antigen consists of cells, prime and boost mice intraperitoneally with $2 \times 10^7$ cells or less. The critical parameter to remember is that fusion should be done 3 days after the last antigen boost.
3. If hyperimmune mice are used as the spleen cell donor, the mice should be rested (generally 3–4 weeks) before receiving the final antigen boost prior to fusion.

## 17.3 FUSION OF NS-1 WITH IMMUNE SPLEEN CELLS

The immediate event in somatic cell hybridization is the fusion of cell membranes, generating multinucleated (generally binucleated) cells or heterokaryons in which the cell membranes of the fusion partners surround a common cytoplasm with two or more nuclei. In a matter of days, synkaryons form when the nuclei fuse and are capable of synchronous mitosis; in the process, a variable number of chromosomes of both fusion partners are lost. With subsequent cell divisions, more chromosomes are lost, but the hybrid cell line eventually stabilizes.

### MATERIALS AND REAGENTS

NS-1 myeloma cells and immune spleen cells (see Section 17.2)
Medium (see Section 17.2A): with FCS (30 ml); without FCS (approximately 100 ml)
37°C water bath
Glass beakers for preparing a makeshift water bath for use in a tissue culture hood
Centrifuge tube, 50 ml, plastic (Corning Glass Works, #25330)
Bench top centrifuge
Pipettes, 1 ml and 10 ml
Stopwatch (optional)
96-well cluster dish (Costar, #3596; see comment 2)
50% solution of polyethylene glycol (PEG) 1500 (BDH Chemicals): PEG 1500 comes as large waxy chunks. It should be odorless and white. Cut away and discard any discolored material. Prepare 50% PEG by the following procedure: Weigh 20–50 g of PEG 1500 in a 100-ml glass reagent bottle and steam autoclave for 20 minutes at 121–132°C. As the PEG cools but before it solidifies, add a volume of RPMI 1640 (20–25 ml) equal to the number of grams of PEG autoclaved. Mix the solution thoroughly. Store the reagent at room temperature. During storage, the 50% PEG solution becomes very alkaline; however, this does not seem to affect the PEG as a fusion agent and nothing should be done to alter the pH.

## PROCEDURE

The following description of the fusion procedure takes into account several characteristics of the 50% PEG solution: (1) The PEG solution is hypotonic; (2) proteins precipitate in 50% PEG; and (3) cells are damaged by PEG treatment. The entire procedure takes 6–7 minutes.

1. Warm 30 ml of medium with FCS and 20 ml of serum-free medium to 37°C. Also, warm the 50% PEG solution to 37°C.
2. Make a 37°C water bath using two glass beakers. Place one beaker of water within a larger beaker, also filled with water. Keep this at 37°C until needed.
3. Harvest the NS-1 cells and wash once with serum-free medium at room temperature. Remove spleens from primed mice, as described in Section 1.2. Prepare a cell suspension and wash 3 times in serum-free medium at room temperature.
4. Mix together $1.5 \times 10^8$ NS-1 cells and $1.5 \times 10^8$ immune spleen cells in a 50-ml centrifuge tube and centrifuge the mixture at 400 $\times$ *g* for 10 minutes at room temperature to form a tight pellet.
5. Remove all supernatant from the pellet and keep the tube at 37°C for further manipulations in the makeshift water bath.
6. Using a 1-ml pipette, add 1 ml of warm 50% PEG over a one-minute period. Gently *stir* the cell pellet with the tip of the pipette as the PEG is being added. Do not pipet the cell suspension. (A stopwatch is helpful to keep track of time.)
7. Continue to *stir* the cell pellet for an additional minute. The goal is to expose the cells to the PEG while maintaining as much cell contact as possible. The cell suspension should look like homogeneous clumps of cells.
8. With the same 1-ml pipette, take one minute to stir in 1 ml of serum-free medium that has been warmed to 37°C. Slow addition of warm medium serves to gradually dilute the PEG without lysing the cells.
9. Repeat step 8.
10. Finally, with a 10-ml pipette, stir in 7 ml of 37°C serum-free medium over 2–3 minutes. Continuous stirring motions should be used. (Pipetting the cell suspension must be avoided as this disrupts the cells.)
11. Centrifuge the suspension at 400 $\times$ *g* for 10 minutes at room temperature and remove the supernatant.
12. Fill a 10-ml pipette with 37°C medium with FCS and aim the tip of the pipette at the cell pellet. By releasing the medium directly at the cell pellet and stirring some with the pipette, a suspension of fine cell clumps is obtained.
13. Add an additional 20 ml of warm medium with FCS and swirl the tube to suspend the contents.
14. Avoiding excessive pipetting, plate 0.1 ml of this suspension ($10^6$ total cells) into each well of three 96-well tissue culture plates. These plates are referred to as the master plates.
15. Place plates into 7% $CO_2$ incubator at 37°C.

1. The ratio of NS-1 to immune spleen cells in the above protocol is 1 : 1; however, a ratio of 1 : 4 (e.g., $4 \times 10^7$ NS-1 cells mixed with $1.6 \times 10^8$ immune spleen cells plated into two 96-well tissue culture plates) has been successfully used. A ratio of 1 : 10 can also be used. Whichever ratio and however many total cells are used in the fusion, the critical parameter is to maintain the initial culture density at $10^6$ total cells per 0.1-ml culture well. The day of fusion is referred to as day 0.
2. Individual cultures in Costar plates are more isolated from each other than they are in similar plates from other suppliers. Costar plates, because of their design, provide the least opportunity for contamination and the best opportunity for eliminating it should it occur. If a plate develops mold contamination, the surest way to prevent its spread to other plates is to discard the contaminated plate. However, if the contamination occurs in one or two wells of a plate that contains valuable cultures, an attempt can be made to salvage the remaining cultures in the plate by rinsing the contaminated well 3 times with 5 M NaOH. To avoid contaminating other plates in the $CO_2$ incubator, isolate the contaminated plate either in a separate incubator or in a humidified culture chamber (Bellco Glass, #7741-10005) until it is clear that the mold has been eliminated.

## 17.4 SELECTION OF HYBRID CELLS (HAT SELECTION)

Cell fusion is a random process and necessitates a means of selecting the desired hybrid cells. Fusion of a population of NS-1 and immune spleen cells results in a mixture of fusion events (NS-1 : NS-1, NS-1 : spleen, and spleen : spleen cells). Selection of NS-1 : spleen cell hybrids is accomplished by culturing the fusion mixture in hypoxanthine-aminopterin-thymidine (HAT) medium. The mechanism of this selection is as follows:

1. Aminopterin (an analog of folic acid) blocks the *de novo* biosynthesis of purines and pyrimidines. To survive in the presence of aminopterin (as in HAT medium), cells must be able to synthesize these nucleotides by utilizing an exogenous source of hypoxanthine and thymidine (provided in HAT medium). They do this via alternate nucleotide biosynthetic pathways aptly called the salvage pathways. (Aminopterin also blocks glycine synthesis, but RPMI 1640 medium supplies enough exogenous glycine to meet this requirement.)
2. NS-1 cells are 8-azaguanine resistant and hence lack an enzyme, hypoxanthine-guanine-phosphoribosyltransferase (HGPRTase), that is required in one of the salvage pathways of nucleotide biosynthesis. NS-1 and NS-1 : NS-1 fused cells are therefore not capable of growing when *de novo* nucleotide synthesis is blocked with HAT medium.
3. Should NS-1 fuse with a normal, albeit antibody-producing spleen cell, the normal cell provides the fused partners with the required enzyme, HGPRTase. This allows the hybrid cell to utilize exogenous hypoxanthine and to grow in HAT medium.
4. There is no positive selection against the growth of infused normal spleen cells and spleen-spleen cell fusions in this scheme; hence it is called half-selection. Passive selection takes place because normal spleen cells have a limited growth potential in culture. By two weeks in culture most spleen cells have died.
5. As a result of this half-selection, the desired NS-1 : spleen cell hybrids are selectively grown.

## MATERIALS AND REAGENTS

### Preparation of 50X HT and HAT Stock Solutions and 1X HT and HAT Media

Supplemented culture medium (see Section 17.2)
Thymidine
Hypoxanthine
Aminopterin

Note: All of the above reagents are available from a number of commercial firms (e.g., Sigma Chemical). Each new batch of reagents should be tested for toxicity before routine use in tissue culture. This is easily done by testing whether the reagent is toxic to a HAT-resistant cell line (e.g., hybridoma cell line developed by HAT selection).

NaOH: 0.1 M (4 g/liter)

### HAT Selection

1X HAT medium
1X HT medium
Needle, 1½ in., 21 or 23 gauge, attached to tubing that is connected to an aspirator
Pasteur pipettes and bulb

## PROCEDURE

### Preparation of 50X HT and HAT Stock Solutions and 1X HT and HAT Media

1. *100X and 50X HT Stock Solutions*
   Prepare a 100X HT solution by dissolving 0.1361 g hypoxanthine and 0.0388 g thymidine in 100 ml double-distilled water warmed to 70–80°C. The 100X HT stock solution is used in preparing the 50X HAT stock solution (see below). To prepare 50X HT stock solution, dilute the 100X solution to 50X with double-distilled water. Sterilize by membrane filtration (Appendix C.2) and store in aliquots at −20°C. (Four-ml aliquots are prepared in our laboratory for addition to 200-ml medium bottles.)
2. *1000X Aminopterin Stock Solution*
   Dissolve 17.6 mg aminopterin in 80 ml double-distilled water. If the aminopterin does not dissolve readily, add several ml of 0.1 M NaOH. Bring volume up to 100 ml with double-distilled water. Store the 1000X aminopterin stock in 10-ml aliquots at −20°C.
3. *50X HAT Stock Solution*
   Combine 100 ml of 100X HT stock, 10 ml of 1000X aminopterin stock, and 90 ml double-distilled water. Sterilize the solution by membrane filtration (Appendix C.2) and store in aliquots at −20°C.

4. 1X HT and 1X HAT Media

Add the 50X stock to an appropriate amount of supplemented culture medium. When thawing the aliquots of 50X stock, some material may come out of solution. However, this material quickly dissolves when the 50X stocks are added to the culture medium.

**HAT Selection**

The following procedure describes a progressive HAT selection scheme. Two objectives are accomplished with this protocol: (a) selection for the growth of hybrid cells; and (b) dilution of immunoglobulin produced by spleen cells. The dilution eliminates some false positive test results in the subsequent assessment of antibody production by hybrid cells.

1. On day 1 (i.e., the day after the fusion), add 0.1 ml of 1X HAT medium to each well. This is done with sufficient accuracy by adding 2 drops of HAT medium with a Pasteur pipette.
2. On days 2, 3, 5, 8, and 11, aspirate off half of the medium from each well and add two drops of fresh HAT medium. After day 11, continue to exchange half of the culture fluid with fresh HAT medium every 3–4 days.

   Aspirating half of the medium from the wells is done visually. The procedure is made easier by placing one edge of the microwell plate onto the edge of another plate thereby having the plate resting at a slight angle. The aspiration needle can then be applied along the upper side of the well to withdraw half of the volume of the culture medium.

## COMMENTS

1. On days 1, 2, and 3 the culture medium will appear quite acidic; thereafter, HAT selection will drastically deplete the cell numbers and the cultures will appear dead. With the aid of an inverted phase-contrast microscope, cells approximately the size of the NS-1 parent can usually be observed growing as colonies among the cellular debris by days 6 and 14 (sometimes, they are not apparent until day 21). Live, growing cells have a distinct appearance with phase-contrast microscopy: bright and translucent. Dead or dying cells appear dark (brown) and opaque. These qualities are not evident without phase-contrast optics.
2. At some point, cells that have grown in HAT medium are transferred to normal medium (RPMI 1640). However, before making this switch, it is necessary for them to grow in HT medium for about one week in order to dilute any remaining intracellular aminopterin. The transfer from HAT medium can be done as early as day 14 but to avoid keeping track of which plates have HAT, HT, and normal media, cells are generally kept in HAT medium until they are transferred from the 96-well plates into 1-ml cultures.
3. As long as the cultures are fed regularly (i.e., with half the medium replaced every 3 days) and not disturbed (i.e., not resuspended), the hybrid cells can remain in the 96-well plates for up to 4 weeks, sometimes even 6 weeks. Successful hybridization will yield growing colonies in every well of the culture plate.

## 17.5 INITIAL SCREENING TO IDENTIFY CULTURES PRODUCING RELEVANT ANTIBODIES

Between two and four weeks after cell fusion (allowing three to four days after the last medium change for antibody to accumulate), the supernates of cultures are harvested using individual pipettes for each culture plate well. The supernates can be tested undiluted or diluted (e.g., 1:5 or 1:10). Dilution of supernates reduces the likelihood of selecting weakly positive wells that may represent marginal antibody production or simply a high assay background. The type of assay used for antibody detection will depend on the goal of the investigator. It is possible to use cytotoxicity or lytic assays; however, these will only detect complement-fixing antibodies and will miss non-complement-fixing antibodies. Solid-phase antibody-binding or cell-binding assays such as those described in Chapter 18 are recommended. When solid-phase antibody-binding or cell-binding assays are used, it is necessary to control for the detection of nonspecific antibody binding (i.e., antibodies that seemingly bind to plastic). Controls for auto-antibodies should be included in work involving alloantigens.

Multiple assays can be done on each supernate to provide an initial characterization of antibody activity; however, such preliminary characterizations may be wasteful of time and effort because many of the positive cultures may fail to yield stable monoclonal antibody-producing cell lines. Whatever detection method is used, the assay must be accurate, reproducible, and rapid, since decisions about which culture wells to save or discard must be made quickly.

## 17.6 TRANSFER TO ONE-MILLILITER CULTURES (TRANSFER PLATES) AND FREEZING OF HYBRID CELLS

The next step after determining which wells are making antibodies of interest is to transfer the cells into 1-ml cultures in 24-well tissue culture plates. This is the first step in expanding the cell lines for cloning and in generating enough cells for frozen stocks. The transfer is accomplished using BALB/c thymocytes as feeder cells; without thymocyte feeders, most cells will not grow when they are transferred into the 1-ml cultures. BALB/c thymocytes can be used regardless of the H-2 haplotype of the donor spleen cells. (Remember NS-1 is of BALB/c origin.)

### MATERIALS AND REAGENTS

Thymocytes (see Section 1.5) from 4–5 week old BALB/c mice (1 thymus/ml of HT medium)
Fetal calf serum (FCS)
Culture medium (see Section 17.2)
HT medium (see Section 17.4)
Dimethyl sulfoxide (DMSO)
24-well tissue culture plates (Costar, #3524; see comment 2, Section 17.3)
Pasteur pipettes and bulb
T flasks
Liquid nitrogen freezer
Freezing vials (Nunc, #N1076-1)

## PROCEDURE

1. Place 0.5 ml of HT medium into each well of the transfer plate.
2. Remove thymuses from mouse donors and prepare a cell suspension. Wash the thymocytes at least 3 times and resuspend them at a concentration of 1 thymus/ml of HT medium. Using a Pasteur pipette, add 1–2 drops of this cell suspension to each well of the transfer plate ($1–2 \times 10^7$ thymocytes per well).
3. Resuspend the contents of each antibody-producing master plate well with a Pasteur pipette and transfer the entire suspension into the transfer well containing the thymocytes and HT medium. Resuspend this mixture and then add back 5 drops of this suspension to the original master plate well. This creates duplicate cultures (a master plate and a transfer cell culture), which protects against losing the new cell line.
4. After 2–3 days, feed these cultures an additional 0.5 ml of HT medium (no additional thymocytes are needed).
5. Two days later, feed the cultures again by first removing as much supernate as possible and adding fresh HT medium.
6. When the cells are nearly confluent (about one week), retest the supernate for antibody activity. Because antibody produced in the master plate is carried over into the transfer cultures, it is important to compare titrations of supernate antibody from the master plate and the transfer plate to determine whether the transferred cells are continuing to produce antibody. Residual antibody from the master plate may produce false positive results. (Transferred cells may lose the ability to produce because of a loss of chromosomes, the overgrowth of the culture by non-producing hybrid cells, or overgrowth by hybrid cells producing antibody of another specificity.)
7. If the culture continues to produce the desired antibody, then clone the cell line immediately. A small fraction of the 1-ml culture is used for this purpose; the procedure is described in the next section. If the antibody-producing wells are not numerous, it is possible to clone directly from the master plate. However, cloning from cultures that continue to produce antibody after transfer reduces the likelihood of working with the less stable cell lines.
8. After removing samples for cloning, expand the remaining cultures in order to have enough cells to freeze and store in liquid nitrogen. Do this by placing the remaining portion of the 1-ml culture into a small T flask containing 5 ml of fresh medium (normal medium may be used at this stage). When this culture becomes dense (approximately $1–2 \times 10^5$ cells/ml), transfer to a larger tissue culture flask and feed 15 ml of medium.
9. Generally for each 10 ml of culture, one vial of cells is frozen. Centrifuge 10 ml of a growing culture containing a total of approximately $2 \times 10^6$ cells. Resuspend the cells in 0.5 ml of 90% FCS, 10% DMSO. Transfer the cells to a freezing vial and immediately begin to freeze the cells either by placing the vials into the *gas phase* of a liquid nitrogen freezer or by insulating the vials with wrapping material and placing them in a −70 C freezer; transfer the vials to a liquid nitrogen freezer after 24 hours.
10. To retrieve frozen cells, thaw the cells quickly and wash immediately with 10 ml of medium. After this wash, resuspend the cells in 10 ml of medium in a T flask and place in the incubator. Expand thawed cells slowly. Initially split the cultures 1:2 to maintain high cell density (approximately $1–2 \times 10^5$ cells/ml) until they fully recover from being frozen.

## 17.7 CLONING BY LIMITING DILUTION*

(11)

### MATERIALS AND REAGENTS

Thymocytes
Culture medium (see Section 17.2)
HT medium (see Section 17.4), for use only when cloning from master plate
Acridine orange-ethidium bromide (AO/EB), for determining cell viability (Section 1.15)
96-well tissue culture plates (Costar, #3596; see comment 2, Section 17.3)

* Specific antibody-producing hybrid cells can be selected and cloned with the fluorescence-activated cell sorter (FACS; Becton, Dickinson FACS Division). Antigen-coated fluorescent microspheres (0.9 μm) are used to stain hybrid cells producing antibody reactive with the antigen. Using the FACS with some electronic modifications (Parks et al. 1979), antigen-binding hybrid cells are sorted and individually deposited into the wells of a 96-well culture plate with thymocytes ($10^6$ cells/well) as feeder cells. When the appropriate antigens are not readily coupled to the microspheres, the FACS can be used to clone cells on the basis of viability. This method has been successfully used to "sorter clone" antibody-producing hybrid cells reactive with mouse immunoglobulin allotypes as early as 16 days after hybridization.

### PROCEDURE

The cloning medium consists of $10^7$ thymocytes per ml of 15% FCS in RPMI 1640. (If cloning is done directly from the master plate, HT medium is used; see comment 2, Section 17.4.) The thymocytes act as carrier cells in diluting the hybrid cells and also as feeder cells in culture. Again BALB/c thymocytes are used. The objective is to plate 36 wells of a 96-well tissue culture plate with an average of 5 cells/well, 36 wells with an average of 1 cell/well, and the remaining 24 wells with an average of 0.5 cells/well. One of these plating concentrations will yield wells with monoclonal growth. The dilutions are carried out as follows:

1. Remove samples from the 1-ml cultures and determine the concentration of viable cells by staining with AO/EB (Section 1.15). Trypan blue which is commonly used to determine viability, should be avoided in this instance because the bovine serum albumin in the medium will bind the stain and thereby produce misleading results.
2. Dilute a sample of the culture to be cloned so that 230 live hybrid cells are suspended in 4.6 ml of the thymocyte-containing cloning medium. Plate 36 wells of a 96-well plate with 0.1 ml of this mixture. This will leave 1.0 ml of cell suspension. To this, add an additional 4.0 ml of the thymocyte-containing medium and plate another 36 wells with 0.1 ml. Finally, add 1.4 ml of the thymocyte-containing medium to the remaining cell suspension and plate the last 24 wells.
3. At day 5 and again at day 12, feed the cloning plate by adding 2 drops of medium with a Pasteur pipette. By day 14 the clones should be large enough to test. Depending on culture conditions, cloning efficiencies, and counting and dilution errors, one of the three dilutions plated should yield wells with no growth (e.g., if an average of 1 cell/well is plated, 37% of the wells should have no growth). Wells appearing to be monoclonal are then tested for antibody activity.
4. Transfer 6 positive clones into separate 1-ml cultures with thymocytes as described above (Section 17.6, except with normal medium). Test the supernates for antibody at the end of a week.

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5. Transfer at least 2 of the positive clones into 5-ml flasks and further expand the cultures. Freeze cell stocks as soon as possible (at least 6 vials should be frozen for each clone). It is now possible to grow cultures for antibody production as described in Section 17.8.

## 17.9 ANTIBODY PURIFICATION: PROTEIN A-SEPHAROSE COLUMN CHROMATOGRAPHY

It is frequently desirable to purify the antibodies synthesized by hybrid cell lines. Several methods of purifying immunoglobulins have been described in Chapters 11 and 12. A recently developed method, which provides rapid purification of immunoglobulins in a single step, utilizes protein A-Sepharose column chromatography. Mouse $IgG_{2a}$, $IgG_{2b}$, $IgG_3$, some $IgG_1$, and some IgM will bind to protein A (Goding 1978). Ey et al. (1978) describe in detail the use of various buffers to elute different classes of mouse immunoglobulin. Elution is done by lowering the pH of the protein A column. The majority of mouse immunoglobulins bind to protein A at pH 8.1 and elute from the column at pH 4.3, or higher; thus harsh acidic elution can be avoided.

### MATERIALS AND REAGENTS

Protein A-Sepharose CL 4B (Pharmacia Fine Chemicals)
$NaN_3$. Caution: Sodium azide is extremely toxic.
NaCl, $M_r = 58.4$
NaOH, $M_r = 40.0$
Tris (tris (hydroxymethyl) aminoethane), $M_r - 121.1$
HCl, concentrated
Citric acid monohydrate, $M_r = 210.15$
Trisodium citrate dihydrate, $M_r = 294.12$
$Na_2HPO_4$, $M_r = 142.0$
$NaH_2PO_4$, $M_r = 138.0$
Acetic acid, glacial
Glycine-hydrochloride, $M_r = 111.5$
Fraction collector
UV monitor (optional)
Buffers: The molarity of the buffering component may be varied. The important parameter is the pH. Sodium azide may be replaced by any suitable preservative (e.g., pentachlorophenol).

- 0.05 M Tris, 0.15 M NaCl, 0.02% $NaN_3$, pH 8.6: For 1 liter of buffer, dissolve 6.06 g of Tris and 8.76 g of NaCl in 800 ml of distilled water. Add 10 M HCl to pH 8.6 and make up the volume to 1 liter with water.
- 0.05 M phosphate, 0.15 M NaCl, pH 7.0: For 1 liter of buffer, dissolve 2.17 g of $Na_2HPO_4$, 1.35 g of $NaH_2PO_4$, and 8.76 g of NaCl in water to 1 liter; buffer pH should be 7.0.
- 0.05 M citrate, 0.15 M NaCl, pH 5.5: For 1 liter of buffer, dissolve 2.68 g of citric acid monohydrate, 10.96 g of trisodium citrate dihydrate, and 8.76 g of NaCl in water to 1 liter; buffer pH should be 5.5.
- 0.05 M acetate, 0.15 M NaCl, pH 4.3: For 1 liter of buffer, dissolve 6.8 g of sodium acetate and 8.76 g of NaCl in 800 ml of water. Add acetic acid to pH 4.3 and make up the volume to 1 liter with water.
- 0.05 M glycine-hydrochloride, 0.15 M NaCl, pH. 2.3: For 1 liter of buffer, dissolve 5.6 g of glycine-HCl and 8.76 g of NaCl in 800 ml of water; add 10 M HCl to pH 2.3 and make up the volume to 1 liter with water.

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## PROCEDURE

Note: Purification is performed at room temperature.

1. Swell 1.5 g protein A-Sepharose CL 4B in Tris-buffered saline, pH 8.6. Pack resin in a suitable column (bed volume is 5–6 ml).
2. Harvest culture supernate and adjust to pH 8.6 by adding dilute NaOH.
3. Apply culture supernate to the protein A column. Wash column with Tris-buffered saline, pH 8.6. (One liter of culture supernate at pH 8.6 containing 10–60 mg of antibody is easily passed through the protein A column.)
4. Carry out step elution with the buffered saline at pH 7.0, 5.5, 4.3, and 2.3 until the hybrid cell antibody is eluted, avoiding low pH buffers whenever possible. A UV monitor is useful in detecting antibody elution from the column.
5. Pool fractions containing antibody and dialyze using an appropriate buffer (e.g., 0.05 M Tris, 0.15 M NaCl, pH 8.1).
6. Regenerate column by washing with the glycine-HCl-buffered saline, pH 2.3, and equilibrating with the Tris-buffered saline, pH 8.6 (including 0.02% $NaN_3$).

## COMMENTS

1. Sometimes two protein peaks will be eluted from a supernate from a monoclonal cell line. By all physical criteria, the protein from both elution peaks may be identical. A possible explanation for this result is that protein A has two binding sites of different affinities for immunoglobulin (Lancet et al. 1978), hence elution conditions for a single protein species may require solutions of different pH.
2. When the antibody produced by a hybrid cell line does not bind to protein A, it is generally easier to purify the antibody from the sera of tumor-bearing mice by standard procedures. The disadvantage of this approach is the presence of normal serum immunoglobulin. Naturally occurring antibodies, for example, antiviral antibodies, may be co-purified with the hybrid cell antibody when affinity chromatography purification methods are precluded by the nature of the antigen, such as with cell surface antigens.

## 17.10 ANTIBODY CHARACTERIZATION

The monoclonal origins of the antibodies produced by hybrid cell lines must be confirmed by demonstrating the production of homogeneous antibody molecules. When NS-1 is used as the parent myeloma, a monoclonal cell line can produce three species of antibodies, because the cells will synthesize the heavy (H) and light (L) chains of the spleen cell parent as well as the MOPC-21 $\kappa$ chain (K) of NS-1 origin. In the intracellular process of assembling the immunoglobulin, mixed molecules are made and secreted. These occur as the following four-chain species: $H_2L_2$, $H_2LK$, and $H_2K_2$. Of course, antibody activity is limited to the first two species. In the process of selecting antibody-producing clones, it is possible to select, clones that have lost the ability to synthesize the MOPC-21 $\kappa$ chain. These clones would then produce no mixed molecules, and every immunoglobulin molecule would be an identically active species.

The chain composition of the products of hybrid cell lines can be determined by various gel analyses. A description of these techniques is beyond the scope of this chapter. Two particular systems are recommended and references to these techniques are noted: (1) reducing and nonreducing isoelectric focusing (IEF; Williamson 1978); (2) two-dimensional analyses using a nonequilibrium pH gradient and size separation (see Chapter 19).

## 17.11 CELL DISTRIBUTION CENTER

When an investigator generates a hybrid cell line producing an antibody that would have utility and interest to the general scientific community, it is urged that the cell line be made available to other investigators through the Cell Distribution Center at the Salk Institute (P.O. Box 1809, San Diego, CA 92112).

The Center already has available hybrid cell lines producing several anti–I-$A^k$ antibodies (some of which cross-react with I-A antigens of *f*, *r*, and *s* haplotypes), anti-H-s$K^k$, anti-Ig-5a(δ), and anti-Ig-5b(δ); by the time this book is published, it will have even more lines available.

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