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T-cell hybridomas producing hapten-specific suppressor factors

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Summary. We have made several T-T hybridomas which secrete soluble factors capable of suppressing an *in vitro* antibody response to nitrophenol (NP), but not other unrelated antigens.

These factors bind specifically to NP, and express determinants coded for in the I-J region of the mouse major histocompatibility complex. No determinants that cross-react with the constant regions of mouse immunoglobulins are present on the factors.

Three sub-clones originating from the same initial culture well of hybridoma cells secrete factors which carry I-J determinants of different haplotypes. One clone expresses I-J determinants derived from the supppressor cell parent, another expresses I-J determinants derived from the tumour cell parent, and a third expresses both. This correlates exactly with I-J determinants expressed on the cell membrane, and suggests the participation of at least two genes in the determination of suppressor-factor structure.

INTRODUCTION

The technique of cellular hybridization is currently having a major impact in immunology, in the same manner that it has led to significant progress in cell biology (see Ringertz & Savage, 1976). B-cell-derived hybridomas which produce monoclonal antibodies of many different specificities have been generated, and many of the problems of obtaining large quantities of monospecific antisera have been overcome (reviewed Melchers, Potter & Warner, 1978).

With respect to T-cell functions, the technique has not yet had the same impact, despite reports (Kontiainen, Simpson, Bohrer, Beverley, Herzenberg, Fitzpatrick, Vogt, Torano, McKenzie & Feldmann, 1978; Taniguchi & Miller, 1978; Taniguchi, Saito & Tada 1979; Watanabe, Kimoto, Maroyama, Kishimoto & Yamamura, 1979; Hewitt & Liew, 1979; Taussig, Cornvalan & Holliman, 1979) of functional T-cell hybridomas that make detectable quantities of antigen-specific suppressor factors. Functional studies of T-cell hybrids promise a more profound analysis of the nature and function of molecules produced by T cells and this approach is being used in many laboratories.

Chemically-defined haptens have been of much use in defining the combining site of B-cell products, and recently of T-cell receptors (Jack, Imanishi-Kari & Rajewsky, 1977. Krawinkel, Cramer, Imanishi-Kari, Jack, Rajewsky & Makela, 1977a). To examine the nature of the antigen combining site of specific T-cell factors, we have used the nitrophenyl (NP) hapten system, where many genetic markers of the antigen combining site of the antibodies have been detected by Makela and his associates: idiotype markers (Jack *et*

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al., 1977; Karjalainen & Makela, 1978; Karjalainen, 1980) and a heteroclicity marker—a stronger reaction with the related hapten nitroiodophenol (NIP) than with the immunogen NP, (Imanishi & Makela, 1974). NP-specific T cells are known to share these markers as judged by absorption onto hapten immunoadsorbents, and on the basis of anti-idiotype binding (Krawinkel *et al.*, 1977a; Krawinkel, Cramer, Mage, Kelus & Rajewsky, 1977b; Krawinkel, Cramer, Melchers, Imanishi-Kari & Rajewsky, 1978; Cramer, Krawinkel, Melchers, Imanishi-Kari, Ben-Neriah, Givol & Rajewsky, 1979). Methods for the generation and assay of hapten (NP) specific helper and suppressor cells and factors *in vitro* were recently described (Kontiainen & Feldmann, 1980).

In this paper we describe three clones of NP-specific suppressor T cells, derived from the fusion of the AKR T-cell tumour line BW 5147 and NP-specific suppressor cells of C57Bl/10 (B10/ScSn) origin. These NP-specific suppressor factors all bear I-J coded determinants; some clones release factors bearing I-J^b, derived from the suppressor cell parent, whereas others bear I-J^k derived from the tumour parent. This observation indicates that I-region determinants have a different genetic origin from those determining the NP-combining site, which presumably come from the NP-immunized B10 spleen cell parent. This suggests that suppressor factors are controlled by at least two genes, one controlling the antigen-combining site and another controlling the I-J determinants. Implications of these findings for suppressor factor structure and function are discussed.

MATERIALS AND METHODS

Hybridization procedure

B10 (H-2^b) suppressor cells reactive to NP (SC_{NP}) induced *in vitro* as previously described (Kontiainen & Feldmann, 1980) were fused with BW5147 (Goldsby, Osborne, Simpson & Herzenberg, 1977; Kontiainen *et al.*, 1978). This fusion experiment was designated S5. Briefly, 10^8 *in vitro* induced NP-specific suppressor cells and 10^7 BW 5147 cells (a HGPRT-negative AKR thymoma line obtained from R. Hyman) were washed twice in serum-free balanced salt solution (BSS) and were pelleted together at 400 *g*. Polyethyleneglycol (0·5 ml; PEG, BDH, mol. wt 1,500) 50% in BSS, pH 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 a further 5 ml BSS were added drop wise before slowly filling the tube to 20 ml with BSS. The cells were spun at 4000 *g*, the supernatant was discarded and the cells were gently resuspended in 100 ml Dulbecco's minimum essential medium (DMEM; Flow) plus 20% foetal calf serum (FCS). This suspension was dispersed in 2 ml aliquots into 72 wells of Linbro trays (Flow). Twenty-four hours after the fusion, 1·0 ml of medium was removed from each well and replaced by 1·0 ml of HAT medium (DMEM plus 20% FCS containing $1{\cdot}1 \times 10^{-4}$ M hypoxanthine, $1{\cdot}6 \times 10^{-5}$ M thymidine and 4×10^{-7} M aminopterin). This procedure was repeated on the following 2 days. On days 6, 8 and 10 HT medium was used (DMEM plus 20% FCS containing $1{\cdot}1 \times 10^{-4}$ M hypoxanthine and $1{\cdot}6 \times 10^{-5}$ M thymidine). Thereafter (day 13 after fusion) the medium was changed to DMEM plus 10% FCS, and the contents of any Linbro well which showed evidence of cell proliferation within the next 1–2 weeks were subcultured in Linbro wells. Aliquots of supernatants were tested for suppression when the cells achieved confluent growth in the Linbro plates, and and wells from which functional supernatants were obtained were cloned in 96-well Linbro plates, allowed to expand and then transferred to Nunc flasks (50 ml, Nunclon-Delta 1461). Supernatants of these clones were tested and functionally-active clones were grown continuously. Aliquots of these were regularly frozen down in liquid nitrogen. Three such clones, all derived from one well (no.17) of the first Linbro plating survived and were tested extensively (S5.17c5,20,27).

Culture and assay procedures

For the induction of helper and suppressor cells, NP (4-hydroxy-5-nitrophenylacetic acid) coupled on to coliphage T4 as previously described (Makela, 1966) was used (Kontiainen & Feldmann 1980). The optimum dose for the induction of helper cells (HC_{NP}) was 10^5 living (infectious) haptenated-phage particles/ml, and for suppressor cells (SC_{NP}) induction 10^7 infectious haptenated-phage particles/ml, for 4 days *in vitro*. For the generation of helper or suppressor factors, these cells were cultured for an additional 24 hr *in vitro* with the lower dose of NP-T4 (10^5/ml). To assay for help or suppression NP coupled to the terpolymer of L-glutamic $acid^{60}$-L--$alanine^{30}$-L-$tyrosine^{10}$ (GAT Miles-Yeda, Rehovot, Israel; Lot GAT 7) was used with four groups of NP per 50,000 MW (Kontiainen & Feldmann, 1980). The optimum dose of NP-GAT in the co-operative cultures was found to be 2 μg/well. As a specificity control, keyhole limpet

haemocyanin (KLH) and its trinitrophenylated derivative (TNP-KLH, 8 mol of TNP per 1000,000 MW) were used. (KLH was a kind gift from Professor Marvin Rittenberg, University of Oregon, Portland, U.S.A.). The coupling of TNP to KLH was as previously described (Rittenberg & Amkraut, 1966).

Helper and suppressor cell induction, production of helper (HF) and suppressor (SF) factors, and co-operative cultures were performed as previously described, using Marbrook flasks (Kontiainen & Feldmann, 1973; 1976; 1980). Conditions are summarized as follows:

HC_{NP} $1{\cdot}5 \times 10^7$ spleen cells $+10^5$ NP-T4 cultured 4 days *in vitro*
SC_{NP} $1{\cdot}5 \times 10^7$ spleen cells $+10^7$ NP-T4 cultured 4 days *in vitro*
HF_{NP} $0{\cdot}5 \times 10^7$ HC_{NP} $+10^5$ NP-T4 cultured 24 hr *in vitro*
SF_{NP} $0{\cdot}5 \times 10^7$ SC_{NP} $+10^5$ NP-T4 cultured 24 hr *in vitro*.

The co-operative cultures contained 3×10^5 HC, 3×10^6 normal, unimmunized syngeneic spleen cells and NP-GAT (2 μg/well) in the absence or presence of SF_{NP}. As SF shows no genetic restriction in its action (Kontiainen & Feldmann, 1978), SF could be tested with allogeneic HC and B cells. The HC and B cells used were syngeneic with respect to each other. All the co-operative cultures were performed in triplicates. At day 4 of the co-operative culture the anti-GAT antibody-forming cells (AFC)/culture were assayed.

The specificity of SF_{NP} was tested by adding them to the cultures of TNP-KLH primed spleen cells *in vitro*. These cultures contained 3×10^6 primed spleen cells stimulated with 0·02 μg/well of TNP-KLH, in the presence or absence of SF_{NP}. The IgM and IgG (developed by goat anti-μ and sheep anti-γ anti-DNP AFC were assayed on day 6 of culture.

The numbers of anti-GAT and anti-DNP AFC were assayed as described previously using DNP (Kontiainen & Feldmann, 1973) or GAT (Kontiainen & Feldmann, 1980), coupled to sheep red blood cells (SRBC). The numbers of anti-GAT AFC were obtained as: *AFC* using *GAT-SRC* − *AFC* using *$CrCl_3$* treated *RBC*. Especially in groups where no HC were added (background) the numbers of AFC developed using $CrCl_3$-SRBC were often higher than those using GAT-SRC and resulted in 'negative' means of GAT-specific AFC. With unprimed spleens as the B cell source, only IgM AFC were detected in our culture system. Results are expressed as numbers of AFC/10^6 spleen cells added at the beginning of the co-operative culture ± SE.

Immunoadsorptions

To characterize the supernatants of cloned S5.17 clones, 5, 20, 27 the following immunoadsorbents coupled onto Sepharose beads (Pharmacia, Uppsala, Sweden) were used: NP-BSA (NP-bovine serum albumin, 5 mg/g dry weight of beads); KLH (5 mg/g dry weight of beads); and anti-I-J^b or anti-I-J^k (0·5 ml of undiluted antisera/g dry weight of beads). Equal volumes of supernatant and packed beads were incubated for 2 hr at room temperature. The beads were then washed twice in phosphate-buffered saline (PBS), and bound material eluted at 4° with glycine-HCl buffer, pH 2·4. Eluates were immediately neutralized with Tris-base, and dialysed overnight against saline before being used.

Chromosome analysis

The number of chromosomes in S5.17c5, c20, c27 were enumerated by the method described by Fox & Zeiss (1961) and compared with those of BW 5147 and normal mouse spleen cells.

Surface antigens of S5.17

S5.17c5, 20, 27 were tested for the presence of surface antigens Thy1·1, Thy1·2, H-2^k and H-2^b by fluorescence as described by Culbert, Kontiainen, Cecka, Makidondo, Mackenzie, Simpson & Feldmann (1981).

The monoclonal antisera anti-Thy1·1 and anti-Thy1·2 were obtained from Dr P. Lake. Monoclonal antibodies against the products of H-$2K^k$ and H-$2D^b$ were generously provided by Drs G. and U. Hammerling.

Anti-I-J^k serum was prepared by immunizing B10.A(3R) mice with spleen and lymph node cells from B10.A(5R), and anti-I-J^b by the reciprocal immunization, as previously described (Simpson, Kontiainen, Herzenberg, Bohrer, Torano, Vogt, Beverley, Fitzpatrick & Feldman, 1978). Anti I-J^b was extensively absorbed on B10.A(5R) spleen cells, and anti-I-J^k on B10.A(3R) spleen cells before use.

RESULTS

Functional specificity of S5.17 supernatants

The supernatants of the original seventy-two Linbro wells obtained on day 15 after fusion were tested. Fifty of them gave greater than 50% suppression of NP-help, and fifteen greater than 70% suppression.

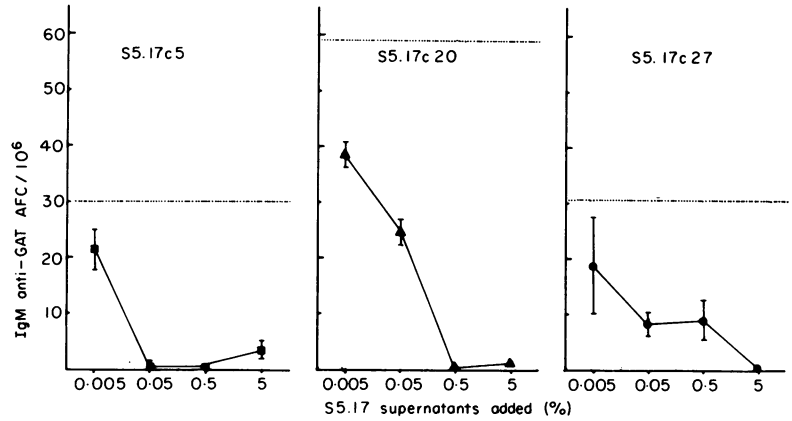

Figure 1. Suppression of response to NP-GAT by S5.17 IgM anti-GAT antibody-forming cells measured at day 4, in separate experiments. Marked suppression required 0·5–5% of S5.17.

Most of the last were subsequently lost, but one of them, from original well number 17, survived.

Preliminary testing revealed that S5.17 was specifically suppressive for NP, as it did not affect the response to TNP-KLH. Thus forty-eight clones obtained from S5.17 were tested for suppressive activity and on the basis of specificity (suppression of NP *v.* KLH) three clones (S5.17c5, c20 and c27) were chosen for further analysis. All of them suppressed NP-specific help by up to 100% at a final concentration of 5 to 0·5% but did not affect responses by TNP-KLH primed spleen cells (Fig. 1). The antigen specificity of the assay used was verified by the criss-cross experiment, as SF_{KLH} did not affect NP-help (Fig. 2).

Characterization of S5.17c5, 20, 27 supernatants

Immunoadsorbtions on antigen columns revealed that the activity of all three clones was removed by NP-immunoadsorbents and was fully recovered in the eluates, confirming the functional specificity of the clones (Figs 2, 3, 4 and 5). The activity was not absorbed by polyvalent anti-mouse immunoglobulin, but was absorbed with anti-I-J preparations. The results with anti-I-J immunoabsorbtions are intrigu-

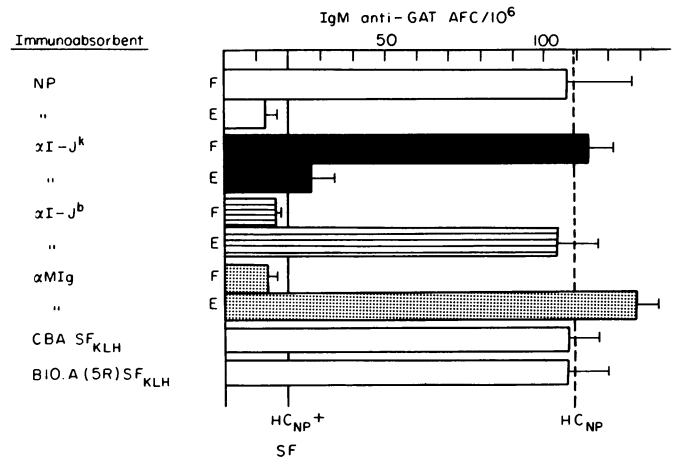

Figure 2. Characteristics of S5.17c27 supernatants. Immunoabsorbent analysis indicates that S5.1727c bears I-J^k determinants, reacts with NP but not with anti MIg. Response is not suppressed by SF_{KLH}.

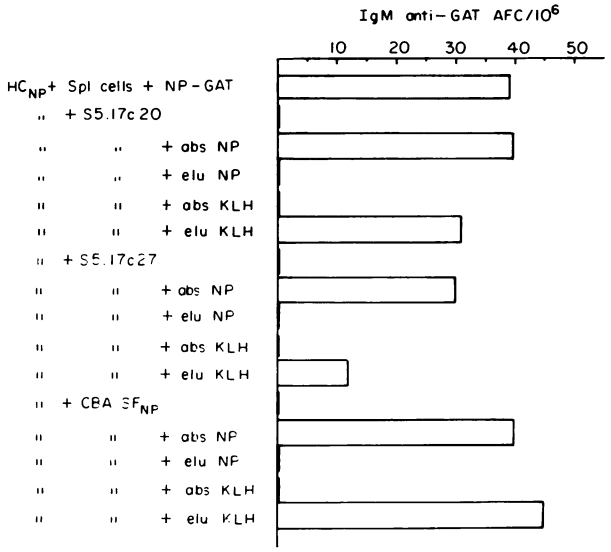

Figure 3. Antigen specificity of S5.17. Clones 20 and 27 supernatants were absorbed with NP or KLH bound to sepharose beads. Binding was significant only with the former.

ing, however. The activity of clone 27 was always absorbed out by anti-I-J^k (Fig. 2), the activity of clone 20 with anti-I-J^b in two out of three supernatants tested in separate experiments, and with anti-I-J^k in

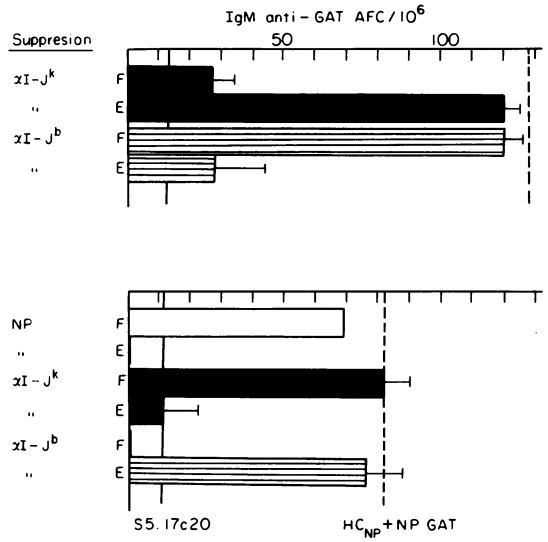

Figure 4. Characteristics of S5.17c20 supernatants. Repeated analysis of different batches of S5.17c20 supernatants indicated that some were reactive with anti I-J^k others with anti I-J^b. Two contrasting experiments are shown.

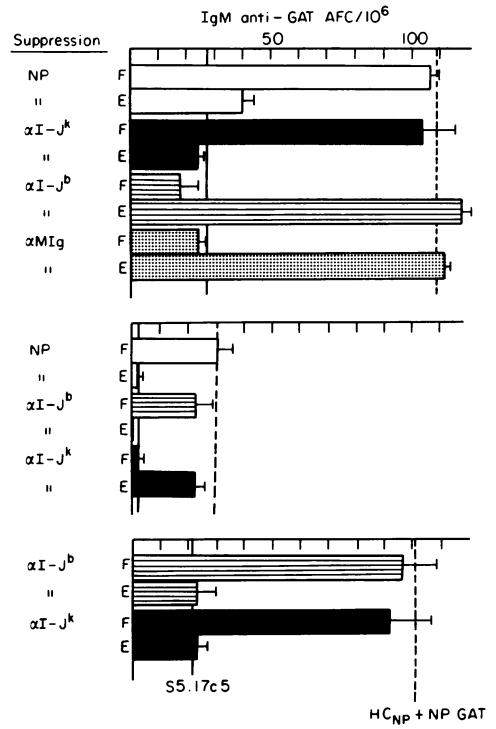

Figure 5. Characteristics of S5.17c5 supernatants. Repeated analysis revealed SF with I-J^k (a) I-J^b (b) or both (c). The latter result was found in only one supernatant, and it is suggestive of molecules being both I-J^k and I-J^b. If a mixture of SF I-J^k and SF I-J^b in different molecules was present, some suppression would have also been noted in the filtrates on the bottom experiment.

the third (Fig. 4). Different preparations of clone 5 supernatant reacted in with either anti-I-J^k or anti-I-J^b, or both (Fig. 5).

Chromosomal analysis of S5.17 clones 5, 20, 27

Karyotypic analysis of normal mouse spleen cells and BW 5147 cells showed a mean number of 40 chromosome per cell in both cases.

The clones of S5.17 showed chromosome numbers in the range 55–72 (Table 1), which indicates these are hybrid cells.

Surface markers of S5.17 clones 5, 20, 27

Fluorescence analysis revealed that all three clones were indeed hybrids, as they stained for both Thy1·1 and Thy1·2, as well as H-2K^k and H-2D^b (Table 2). This was true of all three clones on repeated testing.

Table 1. Karyotypic analysis of S5.17

Cell Source	Chromosome no. (mean ± SD, $n = 20$)
Normal mouse spleen	40·0 ± 0·0
BW5147	40·2 ± 0·5
S5.17c5	67·0 ± 1·0
S5.17c20	67·9 ± 1·1
S5.17c27	63.2 ± 0·9

Table 2. Surface markers of S5.17

	Fluorescent cells			
Antiserum	BW 5147	S5.17c5	S5.17c20	S5.17c27
Mouse serum	9	12	12	13
Anti-Thy1.1	98	99	95	99
Anti-Thy1.2	14	99	98	98
Anti-K^k	93	99	98	98
Anti-D^b	9	99	98	98
Anti-I-J^b	10	37	88	7
Anti-I-J^k	11	89	11	89

et al., 1979). Both antigen-specific and non-specific suppressor cell hybridomas have been reported which make or secrete suppressor factors which resemble those produced by normal cells. The quantities of factors produced by these hybridomas have varied, and on the basis of functional tests been up to ten to 100-fold greater than produced by normal cells, although this has not always been the case. Potentially, these cells may yield sufficient material for the biochemical analysis of T-cell factors and analysis has already started (Taussig *et al.*, 1979; Cecka, in preparation; Pacifico & Capra, 1980) in some laboratories.

We have made hybridomas reactive to NP because the antibody-combining site for this antigen is well characterized both by fine specificity (heteroclicity) and by idiotypic markers (Jack *et al.*, 1977; Karjalainen & Makela, 1978; Karjalainen, 1980; Imanishi & Makela, 1974) enabling possible comparison of T- and B-cell receptors.

The cell line S5.17 produced factors which functioned in the same manner as SF_{NP} derived from normal SC_{NP}. The titres were variable, in keeping with previous experience with hybridomas producing SF_{KLH}. The antigen specificity of the suppressive effect

Table 3. I-J determinants on subclones of S5.17c5

No. of subclones tested	No. I-J^{b+}	No. I-J^{k+}	No. I-J^{b+} and I-J^{k+}
85	5	80	0

Fluorescence with anti-I-J sera showed c20 to be I-J^{b+} and c27 to be I-J^{k+}. S5.17c5 was predominantly I-J^{k+}, but approximately 30% stained with anti-I-J^b.

On recloning by limiting dilution, subclones of S5.17c5 were found to be either I-J^{b+} or I-J^{k+}, but not both (Table 3).

DISCUSSION

There has been much interest in the possibility that T-cell hybridomas may provide the source for a more definite analysis of T-cell receptor and effector function. Several groups, including ourselves, have produced functional T-cell hybrids (Kontiainen *et al.*, 1978; Taniguchi & Miller, 1978; Taniguchi *et al.*, 1979; Watanabe *et al.*, 1979; Hewitt & Liew, 1979; Taussig

identified S5.17 as being useful for further analysis, and it was cloned. Three functional clones were chosen, S5.17c5, S5.17c20, S5.17c27, and their products were partially characterized by immunoabsorption analysis. In keeping with other SF products there was no absorption onto rabbit anti-mouse Ig, but absorption onto the appropriate NP-antigen (Figs 2 and 3). The surprising finding was that anti-I-J^k, but not anti-I-J^b, could absorb out SF activity from some batches of *all* of these three clones. These findings were confirmed with various batches of anti-I-J^k and anti-I-J^b antisera. The specificity of these antisera for suppressor factors has been previously documented (Kontiainen & Feldmann, 1980). The anti-I-J^b reacts with factors produced by H-2^b but not H-2^k derived factors, and vice versa in the case of the anti-I-J^k. One of the clones, c27 yielded a SF which was consistently

absorbed by anti-I-J^k, but others, especially c5 were absorbed by either I-J^b or I-J^k, or both on repeated testing of different batches of SF. Thus the production of two different molecules by c5 (SF_{NP} I-J^k and SF_{NP} I-J^b) cannot be excluded, and the differences between batches may be due to one or other class of the molecule being present at a higher titre in different batches of supernatant. Alternatively it may be that hybrid molecules bearing both I-J's are secreted. Thus the SF bears MHC-coded antigens of either the AKR-parental tumour line or the B10 SC_{NP} parent but presumably had the antigen-combining site coded by B10 SC_{NP} parent, as the idiotypic marker IgV_H NP^b was found in a preliminary experiment (unpublished data).

The expression of I-J coded determinants on the surface of the clones correlated with expression of I-J determinants on their secreted factors, which suggests a direct relationship between secreted and membrane-bound molecules. S5.17c5, which produced SF bearing both I-J^b and I-J^k determinants was stained by both anti-I-J^b and anti-I-J^k. This was due to a mixture of cells, since, on recloning, no 'doubles' have been observed (Table 3).

These results suggest that SF is composed of more than one gene product, with in the case of S5.17 the gene(s) determining antigenic specificity deriving from the C57BL/10 SC_{NP} parent, while other gene(s), such as those controlling I-J determinants come from either the AKR tumour BW 5147 or the parent B10 SC_{NP}. This would imply that there are separate genes controlling at least two structures on the SF molecule. The simplest variant of this idea is that SF has two polypeptide chains (Taniguchi *et al.*, 1979) one containing I-J coded determinants, while the other containing the antigen-combining site and the 'variable' and 'constant' region determinants described elsewhere (Kontiainen & Feldmann, 1979). Thus SF, such as that produced by S5.17c27, would be analogous to a recombinant Ig molecule with H and L chains derived from different haplotypes. This situation has been described for some B-cell hybridoma products (Margulies, Cieplinski, Dharmgrongartama, Gefter, Morrison, Kelly & Scharf, 1977). There are other possible interpretations, such as that the anti-I-J antisera, which often contain anti-viral antibody, may be reacting with viral products in the SF. This is possible at the cell surface, but is unlikely in a SF supernatant product, as not only would it be expected that sera raised in congenic pairs of mice (α-I-J^k and α-I-J^b) should have comparable anti-viral titres, but it would also imply that viral products were incorporated into SF_{NP}. Another possibility, is that both the I-J^k and the combining site are derived from BW 5147. This is unlikely, since preliminary data utilizing anti-idiotype antisera showed that it bore the idiotype of C57Bl origin.

The variable expression of I-J determinants derived from H-2^k or H-2^b on SF_{NP} indicates that the polymorphic determinants of the I-J structures are not involved in the suppressor function of the SF, nor in its antigen specificity. Yet the suppression of tumour cell derived I-J^k on a SF_{NP} indicates that I-J molecules are of major importance in the function of the suppressor factor, perhaps for the reasons (guidance of T-cell subsets) described by Mitchison & co-workers (Czitrom, Mitchison & Sunshine, 1980).

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