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Expression of Spleen Cell Immunoglobulin Phenotype in Hybrids with Myeloma Cell Lines

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Abstract—*Fusions were performed between myeloma cell lines, of mouse and rat origin, and mouse or rat spleen cells. Two statistical methods have been used to measure the proportion of hybrids expressing a spleen cell-derived immunoglobulin phenotype, one of them applicable to cells growing under nonlimiting dilution conditions. The results indicate that there is strong preferential selection for hybrid cell growth with an immunoglobulin-secreting phenotype. The degree of preferential selection is dependent upon the myeloma cell line used and is most marked in the case of the rat myeloma lines. Surviving hybrids seem to originate from fusions of myeloma and spleen B (but not T) cells, but immunoglobulin production is lost more readily in certain combinations.*

INTRODUCTION

When two immunoglobulin-producing cell lines are fused, the resulting hybrids express products of both parental lines (1, 2). When myeloma cells are fused with spleen cells from an immunized mouse, hybrids can be derived which grow in vitro, some of which secrete antibody specific for the immunizing antigen (3). It was observed that the proportion of spleen immunoglobulin-secreting hybrids was higher than expected from the proportion of immunoglobulin-secreting cells in the spleen (3, 4). There appeared to be selective enrichment for the spleen immunoglobulin-secreting phenotype. A proportion of hybrids do not secrete immunoglobulin products of spleen origin, and it was possible that these represented hybrids with spleen T cells. However, the hybrids did not express detectable levels of T-cell-specific cell-surface antigens (5). In contrast, fusion of T-cell lines with spleen cells

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resulted in hybrids expressing T-cell-specific antigens and no secretion of immunoglobulin (6–8).

Recently cell fusions have been performed using a rat myeloma line Y3/Ag 1.2.3. (9). The selective effect of this myeloma for immunoglobulin expression in hybrids seemed even more pronounced than when mouse myelomas were used. The purpose of this study was to determine the proportion of hybrids secreting spleen cell-derived immunoglobulins in a number of rat and mouse cell fusion experiments.

MATERIALS AND METHODS

Cell Lines and Media. All cell lines used were resistant to 8-azaguanine and died in HAT selective medium. None of the lines produces an immunoglobulin heavy chain. For further details of cell lines see Table 1.

Normal medium for cell culture was Dulbecco's modified Eagle's medium (DMEM, Gibco Biocult Ltd., Paisley, Scotland) supplemented with 20% fetal calf serum (FCS, Sera Lab, Crawley Down, Sussex). Batches of foetal calf serum were selected on the basis of tests for their ability to support the growth of newly derived rate and mouse hybrids seeded at low dilution. The fusions described in this paper were carried out over a 6-month period, and two batches of serum were in use, both batches being used for mouse and rat fusions. For selection against parental myeloma cells, hypoxanthine (10 μM), aminopterin (0.4 μM), and thymidine (16 μM) were added to the

Table 1. Parental Myeloma Lines

Name	Species	Strain	Derived from	Immunoglobulin expression
NSI/1Ag4.1[a]	Mouse	Balb/c	P3K[b]	Intracellular K
X63/Ag8.653[c]	Mouse	Balb/c	P3K	None
NSO/U[d]	Mouse	Balb/c	NSI/1Ag4.1	None
Y3/Ag1.2.3[e]	Rat	Lou	R210.RCY3[f]	K
YB2/30Ag20[g]	Rat	(Lou × AO)F_1	Hybrid YB2/3[h]	None

[a]Köhler, G., Howe, S. C., and Milstein, C. (1976). *Eur. J. Immunol.* **6**:292–295.
[b]Horibata, K., and Harris, A. W. (1970), *Expr. Cell Res.* **60**:61–77.
[c]Kearney, J. F., Radbruch, A. D., Liezegang, B., and Rajewsky, K. (1979). *J. Immunol.* **123**:1548–1550.
[d]This is a subline of NSI/1Ag4.1 which does not express the intracellular light chain (Clark, M. R., Wright, B. W., and Milstein, C., unpublished observations).
[e]Galfre, G., Milstein, C., and Wright, B. W. (1979). *Nature* **277**:131–133.
[f]Cotton, R. G. H., and Milstein, C. (1973). *Nature* **244**:42–43.
[g]Wright, B. W., and Milstein, C., unpublished observerations.
[h]YB2/3 is a hybrid myeloma prepared with Y3/Ag1.2.3 cells and a spleen from an AO rat immunized with human complement. Lachmann, P. J., Oldroyd, R. G., Milstein, C., and wright, B. W. (1980). *Immunology* **41**:503–515.

medium (HAT medium) (10). Cultures were maintained in a 10% CO_2, humid atmosphere at 37°C.

Cell Fusions. Fusions were carried out essentially as described by Galfre et al. (11). In the case of mouse cell lines, 2×10^7 cells were fused with 1×10^8 spleen cells, while for rat cell lines 6×10^7 cells were fused with 1×10^8 spleen cells. After fusion the cells were normally distributed equally into 48 × 2-ml cultures (24-well Linbro plates). In the case of limiting dilution analysis the cells were plated in series of 24-well Linbro plates at successive twofold dilutions.

Hybrid growth was observed under the microscope. Generally after two or three weeks of culture there were enough cells for immunoglobulin analysis.

Analysis of Immunoglobulin Production. Approximately 2×10^5 hybrid cells were washed in DMEM and resuspended in 200 μl of − Lysine DMEM, supplemented with [^{14}C] lysine, 5 μCi/ml (Radiochemical Centre, Amersham) and incubated for 18 h at 37°C. Cells were spun down and the supernatants collected.

Supernatants were analyzed by reducing SDS-PAGE on 10% gels according to the method of Laemmli (12) followed by autoradiography for 48 h.

Statistical Analysis. Cells plated at limiting dilution can be analyzed in terms of a Poisson distribution. In the cell fusion experiments described here, hybrid growth often occurred synchronously in all tissue culture wells, indicating multiple hybrid growth. A model based on a multinomial distribution was therefore adopted in order to analyze the data. The model assumes that all hybrids, regardless of their immunoglobulin expression, are equally distributed throughout the tissue culture wells in a random fashion.

The model is as follows:

$$(m + g + o)^n = 1$$

where m = fraction of μ-secreting hybrid clones; g = fraction of γ-secreting hybrid clones; o = fraction of hybrids not secreting a heavy chain; n = number of hybrid clones per culture well.

These cannot be determined directly from the data; however, the following can: (1) $(1 - m)^n$ = proportion of cultures not expressing μ heavy chain; (2) $(1 - g)^n$ = proportion of cultures not expressing γ heavy chain; (3) $(o)^n$ = proportion of cultures not expressing a heavy chain; (4) $(m + g + o)^n - (g + o)^n$ = proportion of cultures expressing μ but not γ heavy chain; (5) $(m + g + o)^n - (m + o)^n$ = the proportion of cultures expressing γ but not μ heavy chain.

The above equations can be solved simultaneously to yield solutions for m, g, o, and n.

In practice, an iterative procedure was used whereby equations 1 and 2 were used with assumed values of n and also with parameters obtained directly from the SDS-PAGE analysis. Solutions for all the remaining equations were then generated and compared with the remaining parameters determined from the SDS-PAGE analysis until the best fit was found. As n is increased, the generated solutions converge on parameters derived from the data and then diverge again as n is increased further.

RESULTS

In order to obtain results which would be generally applicable to most cell fusion experiments involving the production of monoclonal antibodies, the immunoglobulin analysis was carried out on a number of independent cell fusions using various strains and antigens. Details of these fusions, including the cells involved, are given in Table 2. Data collected from the analysis of

Table 2. Details of Cell Fusion Experiments

Fusion	Parental cells	Immunization	Fraction of cultures growing
NN1	NSI/1Ag4.1 × B10.D2 (mouse spleen)	Nylon wool B10 T cells	48/48
NN2	NSI/1Ag4.1 × C3H (mouse spleen)	Nylon wool B10 T cells	48/48
NOA1	NSO/u × Balb/c (mouse spleen)	SRC	48/48
XOA1	X63/Ag8.653 × Balb/c (mouse spleen)	SRC	28/48
YS4	Y3/Ag1.2.3. × DA (rat spleen)	SRC	48/48
YS5	Y3/Ag1.2.3 × DA (rat spleen)	SRC	48/48
YA4	Y3/Ag1.2.3. × DA (rat spleen)	B10 euglobulin precipitate	48/48
YA5	Y3/Ag1.2.3. × DA (rat spleen)	B10 euglobulin precipitate	48/48
YOL1	YB2/3.0Ag20 × Lou (rat spleen)	Yeast tubulin	36/36
NR5	NSI/1Ag4.1 × PVGRTI[1](AGUS) (rat spleen)	AO rat spleen cells	48/48
NR6	NSI/1Ag4.1 × Lew (rat spleen)	F34 rat spleen cells	48/48
YN5	Y3/Ag1.2.3. × B10.D2 (mouse spleen)	Nylon wool B10 T cells	48/48
YN6	Y3/Ag1.2.3. × C3H (mouse spleen)	Nylon wool B10 T cells	48/48

Table 3. Data Accumulated from SDS-PAGE Analysis

Fusion	Number of cultures with particular heavy chain expression				Total cultures analyzed by SDS-PAGE
	No heavy chain	μ heavy chain only	γ heavy chain only	both μ and γ heavy chain	
Mouse myeloma × mouse spleen					
NN1	8	35	0	1	45
NN2	8	14	1	0	23
NOA1	4	4	26	14	48
XOA1[a]	6	4	2	0	12
Rat myeloma × rat spleen					
YS4	0	32	2	12	46
YS5	0	37	1	5	43
YA4	1	21	12	8	41
YA5	0	17	7	3	27
YOL1	3	24	1	0	28
Mouse myeloma × mouse spleen					
NR5	29	15	2	0	46
NR6	22	17	3	3	45
Rat myeloma × rat spleen					
YN5	1	9	9	18	37
YN6	1	5	9	2	17

[a]This fusion is probably at limiting dilution (see Table 2). For technical reasons not all of the growing cultures indicated in Table 2 were analyzed by SDS-PAGE.

immunoglobulin expression by SDS-PAGE are given in Table 3. In order to determine the percentage of hybrid clones which express immunoglobulin heavy chain in each of these cell fusion experiments, the data were analyzed using the statistical analysis described earlier. The analysis yields results which are summarized in Table 4.

Following a fusion, the total population was divided into a number of microcultures. The average number of hybrid clones present in each microculture was calculated in several instances to be one (Table 4). This was somewhat surprising. If the microcultures originate from limiting dilution at a dilution giving only one clone per culture, one expects a good proportion of them to show no hybrid growth. This is not the case (Table 2). This suggests that considerable clonal competition has taken place and that, even at the earliest stage at which we could perform the analysis, the cultures were much simpler than when started.

The expression of spleen cell immunoglobulin phenotype in the hybrids differs considerably depending on the myeloma parent. In the case of mouse myeloma × spleen cell fusions, the percentage of hybrid clones which express an immunoblobulin heavy chain (spleen parent), varies between 39% and 82%. This is in agreement with previously published results (3, 4, 13). The

Table 4. Secretion of Spleen Heavy Chain in Myeloma × Spleen Hybrids[a]

Fusion	Parental myeloma	Average number of hybrid clones per culture	Hybrid clones which secrete Ig heavy chain (%)
Mouse myeloma × mouse spleen			
NN1	NSI/1Ag4.1	1	82
NN2	NSI/1Ag4.1	1	65
NOA1	NSO/u	5	39
XOA1	X63/Ag8.653	1	50
Rat myeloma × rat spleen			
YS4	Y3/Ag1.2.3	2	95
YS5	Y3/Ag1.2.3	1.6	99
YA4	Y3/Ag1.2.3	1.5	92
YA5	Y3/Ag1.2.3	1.2	99
YOL1	YB2/3.0Ag20	1	90
Mouse myeloma × rat spleen			
NR5	NSI/1Ag4.1	1	37
NR6	NSI/1Ag4.1	4	16
Rat myeloma × mouse spleen			
YN5	Y3/Ag1.2.3	3	70
YN6	Y3/Ag1.2.3	1.3	88

[a] Average number of hybrid clones per culture and percentage of clones which secrete immunoglobulin heavy chain obtained by application of statistical method described in text to the data in Table 3.

equivalent values for rat myeloma × rat spleen are considerably higher (between 90% and 99%). This implies that most rat × rat hybrids secrete spleen cell-derived immunoglobulin. In interspecies fusions the use of mouse myelomas and rat spleen cells results in a lower relative yield of immunoglobulin-secreting hybrids (a point which had not been noticed previously, ref. 14, 15), whilst rat myeloma and mouse spleen cells give a much higher relative yield of immunoglobulin-secreting hybrids. However, the relative yield using the rat myeloma as parental line is marginally lower when the spleen is of mouse rather than of rat origin.

To verify these results with the rat myeloma, fusion experiments were performed at limiting dilution permitting an independent check on the validity of the statistical model as applied above. The results are summarized in Table 5.

Fusion YH4 demonstrates that the rat line Y3/Ag 1.2.3, when fused with rat spleen cells, gives rise to hybrids, a very high proportion of which secrete immunoglobulin heavy chain. This result is in agreement with those given in Table 4.

The line YB2/3.O Ag 20 is derived from a hybrid line from a fusion between Y3/Ag 1.2.3 and an AO rat spleen (Table 1). Hybrids between this line and rat spleen cells (fusion YOH1) have a lower relative expression of

Table 5. Limiting Dilution Analysis of Spleen-Derived Immunoglobulin Secretion

Fusion	No. of spleen cells/culture	Growing cultures as fraction of total	Fraction of cultures expressing heavy chain	Fraction of cultures expressing light chain[a]	Percentage of clones secreting spleen immunoglobulin[b]
YH4[c]	2×10^6	24/24	24/24		
	1×10^6	24/24	24/24		
	5×10^5	15/24	15/15		>93
	2.5×10^5	12/48	12/12		>97
YOH1[d]	2×10^6	24/24	20/24	21/24	
	1×10^6	21/24	16/19	18/19	83
	5×10^5	11/24	6/10	7/10	63
	2.5×10^5	5/48	2/3	3/3	>79
YOH2[e]	2×10^6	24/24	18/24	19/24	
	1×10^6	18/24	10/17	12/17	54
	5×10^5	15/24	7/13	8/13	49
	2.5×10^5	9/48	1/6	2/6	31

[a]These refer to immunoglobulin chains from the parental spleen cells. Light chains can be detected in the absence of a myeloma light chain.
[b]Percentage of clones secreting spleen-derived immunoglobulin corrected for statistical coincidence using the Poisson distribution.
[c]YH4 was a fusion between Y3/Ag1.2.3 and the spleen from an unimmunized Lou rat.
[d]YOH1 was a fusion between YB2/3.0Ag20 and the spleen from an unimmunized Lou rat.
[d]YOH2 was a fusion between YB2/3.0Ag20 and the spleen from an unimmunized Balb/c mouse.

spleen cell immunoglobulin heavy chain than Y3/Ag 1.2.3 (see also fusion YOL1, Table 4). When the fusion is with mouse spleen cells (fusion YOH2), the relative expression of immunoglobulin heavy chain is even lower.

DISCUSSION

From early on it was observed that the immortalization of antibody production was accompanied by a selective enrichment of antibody secretion (3, 4). Typically spleens which contained less than 1% of plaque-forming cells gave rise to hybrid populations containing up to 10% of clones secreting the corresponding antibody. This selection probably includes several components. One may be the preferential survival of hybrids with actively dividing spleen cells, i.e., those triggered by antigenic stimulation. In addition the myeloma is capable of producing hybrids with nonsecreting B-cell lymphomas, which actively secrete the lymphoma immunoglobulin (16, 17, M.R. Clark, unpublished observations). In hyperimmune spleens there are a number of cells which synthesize but do not secrete substantial amounts of antibody. Such cells may give rise to actively secreting hybrids. In addition, there seems to be a less obvious but more interesting phenotypic selectivity. It appears that hybrids between myelomas and normal non-B cells may not be successful in getting established as permanent lines (5, 18), although in myeloma × spleen cell fusions there is a population of hybrids which do not express a spleen immunoglobulin the characteristic markers of B cells.

The attempts to define the proportion of hybrid clones, generated in myeloma × spleen cells made before (4, 13), were based on limiting dilution analysis statistics. A statistical analysis described in this paper permits the study of cultures containing multiple clones.

The data presented show that the rat line Y3/Ag 1.2.3, when fused with rat spleen cells, gives rise to hybrids practically all of which secrete spleen cell-derived immunoglobulin. This is in contrast with observations in the mouse system where a large fraction of hybrids does not secrete any immunoglobulin. There are several possible explanations for this difference. It could be argued that the difference reflects differential survival of hybrids of mouse or rat myelomas with non-B (e.g., T) cells. Alternatively activation of immunoglobulin expression may occur in hybrids between the rat myeloma and non-B cells. Activation of T-cell-specific markers has been reported in fusions of T lymphomas with non-T cells (19, 20). Our preferred explanation is that successful fusion is restricted to B cells but that fusion with some myelomas may not always lead to active secretion of the spleen immunoglobulin. Instability and clonal competition in the early stages of hybrid growth results in the loss of expression in some cases more than in others. This

is supported by the observation in fusions YOH1 and YOH2, where the number of cultures expressing immunoglobulin light chains is greater than the number of cultures expressing immunoglobulin heavy chains. It has been previously observed that the order of chain loss in hybrids almost invariably starts with heavy chain followed by light chain (21). Thus it seems likely that at least some of the YB2/3.O Ag2O hybrids which do not secrete immunoglobulin heavy chain result from loss of expression. The stability of spleen parental immunoglobulin expression in hybrids derived from Y3/Ag 1.2.3 may be not so strongly manifest in YB2/3.O Ag 2O so that hybrids derived with it have a higher proportion of "negatives."

It has also been observed that the Y3/Ag 1.2.3 myeloma κ chain is particularly stable. Attempts using several techniques to obtain a cell line directly from Y3/Ag 1.2.3 which no longer expresses this κ chain have so far proven unsuccessful (B.W. Wright and C. Milstein, unpublished observations). Mutants of this nature have been observed quite frequently in the mouse line P3-X63 Ag 8 (22). For example, the mouse nonproducer line NSO/U arose spontaneously during prolonged culture of the κ-producing cell line NS1/1 Ag 4.1 (M.R. Clark, B.W. Wright, and C. Milstein, unpublished observations).

To interpret results of interspecies fusions it is necessary to include preferential loss of particular chromosomes in such cases. It has been shown that, at least in the case of X63 Ag 8 $\times$ rat spleen fusion there is preferential loss of rat chromosomes but that rat antibody expression is retained because the rat chromosomes which carry the immunoglobulin genes are relatively stable (23). Similar analysis has not yet been carried out for rat myelomas fused with mouse spleen cells.

In conclusion we suggest that when myeloma cells are fused to a mixed population of cells, such as spleen cells, the permanent established hybrids are those derived from fusions with B cells. The proportion of established hybrids which retain the spleen immunoglobulin phenotype is largely defined by the parental myeloma. Interspecies fusions, however, lower this proportion.

It should be noted that whatever the explanation for the differences in immunoglobulin expression shown here, all of these cell lines can be successfully used for the derivation of hybrid lines secreting monoclonal antibodies. Also, once such lines have been properly isolated and cloned, they are stable for immunoglobulin expression.

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