

## LOSS OF SECRETION IN MOUSE-HUMAN HYBRIDS NEED NOT BE DUE TO THE LOSS OF A STRUCTURAL GENE\*

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Production of mouse human hybrids by fusion of mouse myeloma cells with human lymphoid cells has been well documented since the early studies of Schwaber and Cohen (1, 2). These hybrids do, however, lack stability and display a preferential loss of human chromosomes in the days after fusion (3) in a manner that appears to be nonrandom (4, 5). Their chromosomal instability has proved valuable for gene mapping studies, including experiments that have resulted in the provisional assignment of human heavy chain genes to chromosome 14 (5-7). On the other hand, the preferential loss of human chromosomes has made it difficult to use the hybrid lines for sustained production of human immunoglobulin (Ig) (8, 9). Moreover, the problem has not yet been solved by substituting myeloma cell lines of human origin and, although several groups have successfully produced human-human hybrids (10-12), long-term antibody-secreting cell lines have not to our knowledge been reported.

We describe here three mouse-human lines derived from a single fusion between the mouse myeloma line NS-1 and human tonsillar lymphocytes. One of these lines has continued to secrete human Ig over a 2-yr period in culture. The other lines were less stable and eventually stopped secreting Ig. Nonsecreting lines were examined using isoenzyme assays to determine whether chromosome 14 had been lost. Because the majority of nonsecreting hybrids still had chromosome 14, and therefore appeared to retain the structural genes for human Ig, they were stimulated with mitogens in an attempt to regain Ig secretion. Lipopolysaccharide (LPS)<sup>1</sup> stimulation of three nonsecreting cell lines resulted in sustained Ig production, indicating that the cessation of Ig secretion in those hybrid cells may be caused by factors other than loss of structural genes. The ability to regain Ig secretion through mitogen stimulation suggests that mouse-human hybridomas may still be of potential value as a source of human monoclonal antibody.

### Materials and Methods

*Cell Culture.* NS-1 cells were grown in Dulbecco's modified Eagle's medium (DME), (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 10% fetal calf serum (FCS) (Flow Laboratories, Stanmore, New South Wales, Australia) and antibiotics (penicillin

\* Supported by grants from the Clive and Vera Ramaciotti Foundations and the Cancer Research Committee, University of Sydney.

<sup>1</sup> *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HAT, hypoxanthine, aminopterin, thymidine; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PWM, pokeweed mitogen; RIA, radioimmunoassay; SDME, supplemented Dulbecco's modified Eagle's medium.

100 IU/ml and streptomycin 100  $\mu\text{g/ml}$ ). After fusion, hybrid cells were grown in DME containing  $2 \times 10^{-4}$  M hypoxanthine,  $8 \times 10^{-7}$  M aminopterin, and  $3.2 \times 10^{-5}$  M thymidine (HAT) with FCS and antibiotics as above. Established hybrid lines were cultured in supplemented DME (SDME), DME with added Hepes (2.38 g/liter) and glucose (3.5 g/liter). Tonsillar lymphocytes were grown in RPMI 1640 containing 10% FCS, penicillin and streptomycin as above, and gentamycin (50  $\mu\text{g/ml}$ ). Macrophage-conditioned medium consisted of SDME-10% FCS taken from flasks in which mouse peritoneal exudate cells (initial concentration  $2.5 \times 10^5$  cells/ml) had been grown for 3-4 d.

*Cell Fusion and Hybrid Selection.* Tonsillar lymphocytes were prepared as described previously (8) and cultured for 4 d in flasks at  $2.5 \times 10^6$  cells/ml with a 1:200 dilution of stock pokeweed nitrogen (PWM) (Gibco Laboratories). Cultured tonsillar lymphocytes ( $10^8$  cells) were fused with  $10^7$  P3/NSI/1-Ag4-1 cells (NS-1) in the presence of polyethylene glycol-4000 (Ajax, Sydney, Australia). After fusion, cells were incubated at  $2 \times 10^6$  cells/ml in a 75-cm<sup>2</sup> flask for 12 h, and then distributed in 48  $\times$  2-ml cultures in DME-10% FCS-HAT medium. Normal mouse peritoneal exudate cells were added at a concentration of  $1 \times 10^5$  cells/ml.

*Cloning.* Hybrid cells were cloned by limit dilution in 96-well microtiter plates (Linbro Chemical Co., Hamden, CT).

*Cell Culture with LPS.* Hybrid cells were grown in 2-ml cultures in SDME-10% FCS with 25% macrophage-conditioned medium at an initial concentration of either  $1 \times 10^4$  or  $5 \times 10^4$  cells/ml. LPS from *Salmonella typhimurium* (Sigma Chemical Co., St. Louis, MO) was added at concentrations between 5 and 50  $\mu\text{g/ml}$ .

*Radioimmunoassay.* Supernatants from hybrid cell cultures were screened for production of human Ig by solid-phase radioimmunoassay (RIA) in polyvinylchloride microtiter plates (Cooke Laboratories, Dynatech Corp., Alexandria, VA). In the initial screening of hybrid cultures, goat IgG to human Ig (Oxford Laboratories, Foster City, CA) was bound to the plate, and human Ig in culture supernatants was detected with an <sup>125</sup>I-labeled F(ab')<sub>2</sub> fragment of goat antiserum to human Ig (Oxford Laboratories). In subsequent assays, rabbit IgG to human IgG (Dako, Copenhagen, Denmark) was bound to the plate, and human IgG production by established hybrid lines was detected in an inhibition assay using <sup>125</sup>I-labeled affinity-purified human IgG (13). All results were quantitated with reference to known standards of human IgG.

*Detection of Histocompatibility Antigens and Human Ig on the Surface of Hybrid Cells.* The presence of surface human IgG and human  $\kappa$  or  $\lambda$  light chains on hybrid cells was detected by direct immunofluorescence using either a fluorescein conjugated F(ab')<sub>2</sub> fragment of goat IgG to human IgG (MN 55318; Kallestad Laboratories, Austin, TX), or fluorescein or rhodamine conjugated F(ab')<sub>2</sub> fragments of burro Ig to human  $\kappa$  or  $\lambda$  light chains (Kallestad Laboratories). All washes and incubations were carried out in RPMI 1640 containing 0.1% NaN<sub>3</sub>.

Histocompatibility antigens were detected by indirect immunofluorescence. Washed cells were incubated for 30 min at 4°C with a 1:500 dilution of 2A1, a mouse monoclonal antibody to HLA A, B, C public specificities (the generous gift of Dr. P. Beverley, University College, London, England) or with a 1:100 dilution of 1203 Y, a mouse monoclonal antibody to H-2<sup>d</sup> histocompatibility antigens (the generous gift of Prof. I. F. C. McKenzie, University of Melbourne, Melbourne, Australia). After incubation, the cells were washed three times and then incubated for 30 min at 4°C with a fluorescein-conjugated F(ab')<sub>2</sub> fragment of sheep anti-mouse Ig (New England Nuclear, Boston, MA). Stained cells were washed three times, applied to microscope slides in 90% glycerol in phosphate-buffered saline (PBS) (pH 8.6), and examined under oil immersion with a Zeiss fluorescent microscope (Carl Zeiss Inc., New York). Over 200 cells were counted on each slide.

*Chromosome Preparations.* Hybrid cells were arrested in metaphase during logarithmic growth by the addition of 1  $\mu\text{l/ml}$  of colcemid (0.05 g/ml in Hank's balanced salt solution) followed by 60 min incubation at 37°C. The cells were then washed, resuspended in 1 ml of medium, and exposed to hypotonic conditions by the addition of 6 ml of 0.075 M KCl. After 15 min, the cells were centrifuged and fixed by four washes in 3:1 (vol/vol) methanol/glacial acetic acid. The cell pellet was resuspended in 0.5 ml 3:1 (vol/vol) methanol/glacial acetic acid, and the suspension was dropped onto cold glass slides and spread by rapid air drying. Slides were stained with Giemsa stain or for centromeric heterochromatin (C-banding) after the method of

Boone and co-workers (14).

*Isoenzyme Analysis for Human Chromosomes 6 and 14.* The presence of human chromosomes 6 and 14 in cloned mouse-human hybrids was sought by assaying for the isoenzyme markers glyoxalase I (chromosome 6) and nucleoside phosphorylase (chromosome 14) (15). Cell lysates from selected secreting and nonsecreting hybrid lines were analysed by starch gel or cellulose acetate electrophoresis followed by enzyme-specific staining procedures using a paper overlay. Normal human lymphocytes and the murine plasmacytoma NS-1 were incorporated as controls in each assay.

## Results

*Production and Characterization of Ig-secreting Hybrids.* Fusion of PWM-stimulated human tonsillar lymphocytes with NS-1 yielded three cloned human Ig-secreting lines designated B1-29, A2-31, and E2-42. These cloned lines varied both quantitatively and qualitatively in the human Ig secreted. All failed to express HLA while retaining the H-2<sup>d</sup> haplotype of the murine parent line (Table I).

Karyological studies carried out on B1-29, the only hybrid line to remain secreting human Ig >18 mo after initial isolation, revealed a mean of 122 chromosomes/cell, more than twice the number of chromosomes found in NS-1. Counts on C-banded metaphase preparations indicated that at least 22 of these 122 chromosomes were putatively of human origin.

*Recloning of B1-29 18 mo After Initial Isolation.* B1-29 was the only hybrid line that continued to secrete human Ig 18 mo after initial isolation. At this stage, it was recloned by limit dilution (Fig. 1 A), and each resulting subclone was assessed for human IgG secretion with an inhibition RIA for human IgG. Nonsecreting clones were defined as cultures with <0.1 µg of human IgG/ml, whereas high-secreting clones were cultures with >0.8 µg/ml human IgG. 403 subclones of B1-29 were assayed, of which 24% were nonsecreting and only 7% were high-secreting clones (Fig. 1 A). Two high-secreting subclones (H1 and H3) were then recloned (Fig. 1 B, C), and this procedure was repeated yet again with H1-4 and H3-4, two high-secreting subclones obtained from the cloning of H1 and H3, respectively (Fig. 1 D, E). The frequency of high-secreting clones was dramatically enhanced by this procedure. All 41 subclones assayed from H3-4 secreted high levels of human IgG (>1 µg/ml), whereas 57% of the 27 clones assayed from H1-4 were high secretors. The recloning of B1-29 produced a panel of nonsecreting and high-secreting subclones that were then

TABLE I  
*Characterization of Three Mouse-Human Hybrid Clones Secreting Human Ig*

Cell line	Doubling time	Secretion of human IgG*	Ig subclass‡	Light chain type§	HLA	H-2 <sup>d</sup>
	<i>h</i>	<i>µg/10<sup>5</sup> cells</i>				
A2-31	34	15.0	γ <sub>1</sub>	ND¶	—	+
B1-29	46	3.7	γ <sub>1</sub>	Human λ	—	+
E2-42	33	0.7	γ <sub>4</sub>	Human λ	—	+
NS-1	22	0	—	Mouse κ	—	+

\* Detected by an inhibition-type RIA using <sup>125</sup>I-labeled, affinity-purified human IgG.

‡ Determined by immunodiffusion using subclass specific goat anti-human antisera.

§ Determined by direct immunofluorescence using fluorescein- or rhodamine-conjugated burro anti-human κ or λ.

|| Determined by direct immunofluorescence (see Materials and Methods).

¶ Not done.

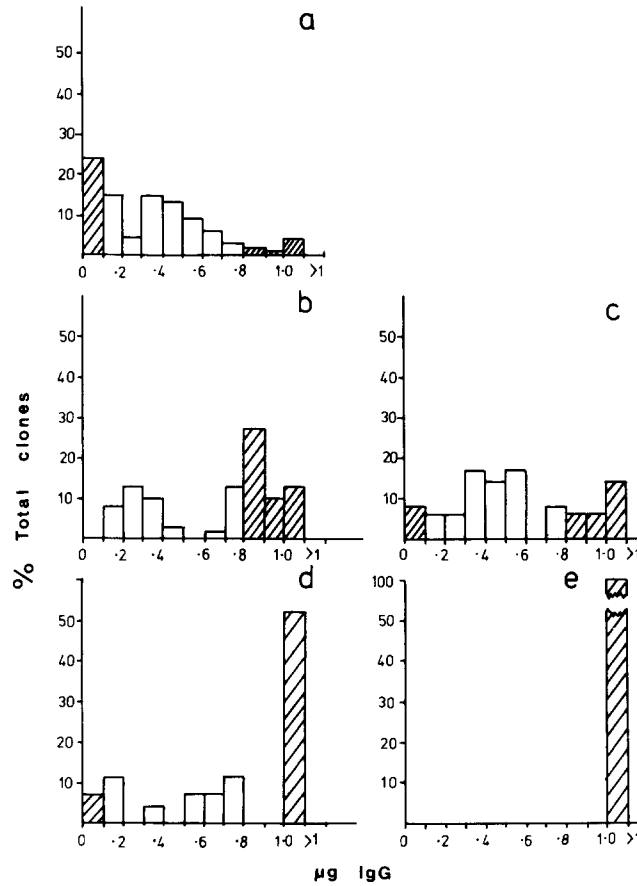


FIG. 1. The frequency of human Ig secretion in B1-29 after recloning. (a) 403 subclones from B1-29; (b) 60 subclones from H1 and (c) 36 subclones from H3 (H1 and H3 were two high-secreting subclones derived from B1-29); (d) 27 subclones from H1-4, a high-secreting subclone derived from H1; (e) 41 subclones from H3-4, a high-secreting subclone derived from H3.

examined for the presence of surface Ig and chromosomes 6 and 14.

*Surface Ig on Secreting and Nonsecreting Subclones of B1-29.* Selected subclones of B1-29 were examined by direct immunofluorescence for the presence of surface human IgG (Table II). Only 2 out of 30 nonsecreting subclones of B1-29 had detectable surface human IgG. Loss of secretion by these mouse-hybrid lines thus tended to correlate with loss of surface Ig. The high-secreting lines expressed variable levels of surface human IgG, with between 0 and 55% of total cells counted showing fluorescent staining.

*Detection of Human Chromosomes 6 and 14 in Mouse-Human Hybrids.* The three original mouse-human hybrid lines and selected high-secreting and nonsecreting subclones of B1-29 were examined for the presence of human chromosomes 6 and 14 by assaying for glyoxalase I and nucleoside phosphorylase, respectively. All three original lines lacked human glyoxalase I (Table III), which implies that human chromosome 6 is absent. B1-29 and E2-42 contained human nucleoside phosphorylase, the marker for chromosome 14, but this was absent in A2-31. At this time, B1-29 was the only cell

TABLE II  
*Mouse-Human Hybrids: Surface and Secreted Human IgG*

Cell line	Description	Percent cells with surface human IgG*	Secretion of human IgG‡ <i>µg/ml</i>
B1-29	Original cell lines	25%	0.6
A2-31		0%	0
E2-42		11%	0
L1 to L7	32 nonsecreting subclones of B1-29	0%	0
L10 to L32		0%	0
L8		1%	0
L9		8%	0
H1	high-secreting subclones of B1-29	14%	1.3
H2		11%	0.7
H3		6%	0.8
H4		8%	1.6
H5		2%	0.8
H6		55%	1.3
H7		0%	2.0

\* Determined by direct immunofluorescence with a fluorescein-conjugated F(ab')<sub>2</sub> fragment of goat antiserum to human IgG.

‡ These values were determined using an inhibition-type RIA 18 mo after the three original cell lines had first been isolated.

TABLE III  
*Species of Nucleoside Phosphorylase in Mouse-Human Hybrids*

Cell type	Nucleoside phosphorylase (chromosome 14)	Glyoxalase I (chromosome 6)
Human tonsil	Human	Human
NS-1	Mouse	Mouse
B1-29*	Human and mouse	Mouse
E2-42	Human and mouse	Mouse
A2-31	Mouse	Mouse
B1-29 nonsecreting subclones	Human and mouse (13)‡	Mouse (4)
B1-29 secreting subclones	Human and mouse (7)	Mouse (3)

\* B1-29 was the only original line still secreting human IgG when assays for isoenzymes were carried out.

‡ The numbers in brackets refer to numbers of B1-29 subclones examined.

line still secreting human Ig. All subclones of B1-29 examined had human nucleoside phosphorylase but lacked human glyoxalase I, regardless of whether they had stopped secreting Ig or not.

*Retrieval of Human IgG Secretion in Nonsecreting Hybrids by Stimulation with LPS.* Because nonsecreting hybrids appeared to retain chromosome 14, an attempt was made to retrieve Ig secretion. Two of the original lines, A2-31 and E2-42, which had lost

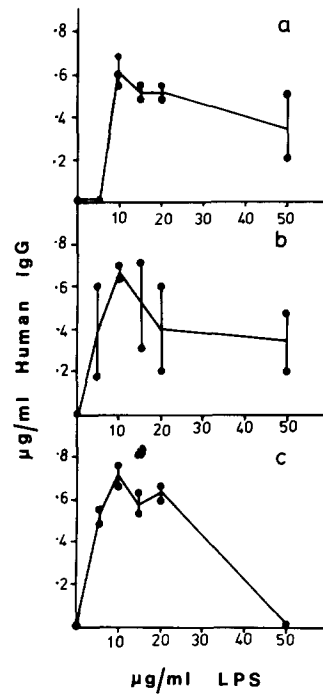


FIG. 2. The effect of LPS concentration on human Ig secretion in three mouse-human hybrid lines after 24 h in culture. Initial cell concentration was  $1 \times 10^4$  cells/ml. (a) B1-29; (b) E2-42; (c) A2-31.

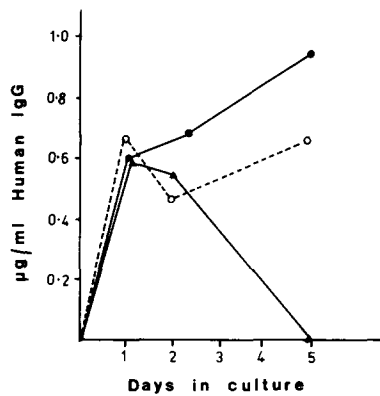


FIG. 3. The effect of 10 µg/ml of LPS on human Ig secretion for 5 d in culture. The three mouse-human hybrid lines were set up at an initial concentration of  $1 \times 10^4$  cells/ml. ●, B1-29 L8; ○, E2-42; ▲, A2-31.

secretion, and B1-29-L8, a nonsecreting subclone of B1-29, were grown in 2-ml duplicate cultures at an initial concentration of either  $1 \times 10^4$  or  $5 \times 10^4$  cells/ml in the presence of 5–50 µg/ml LPS. Cells from these three lines not only did not secrete, but also had little or no surface human IgG (Table II).

Samples of culture supernatants were taken on days 1, 2, and 5 and assayed quantitatively for the presence of human IgG. All cultures set up at  $1 \times 10^4$  cells/ml responded to LPS by secreting human IgG, whereas there was little response in

cultures set up at  $5 \times 10^4$  cells/ml. Human IgG secretion was readily detectable after 24 h culture (Fig. 2) and was greatest in the presence of 10–20  $\mu\text{g/ml}$  LPS, reaching  $>0.7 \mu\text{g/ml}$  human IgG. Thus, the response to LPS was highly dose dependent. Secretion of human IgG by B1.29.L8 and E2.42 continued until at least day 5 of culture, whereas A2.31 peaked between days 1 and 2, and IgG could not be detected on day 5 (Fig. 3). This did not seem to be caused by death of cells in the cultures, as viability on day 5 was normally  $>80\%$ .

### Discussion

This paper describes the features of three mouse-human hybrid lines secreting human Ig of unknown specificity. They were obtained from a fusion between the mouse myeloma line NS-1 and human tonsillar lymphocytes that had been stimulated for 4 d *in vitro* with PWM. The hybrid lines secreted human IgG of the  $\gamma_1$  or  $\gamma_4$  subclass with  $\lambda$ -type light chains (Table I). One line, B1-29, has continued to secrete human IgG for 2 yr in culture, but the other two lines lost secretion after several months.

Loss of secretion of human Ig is a common occurrence in mouse-human hybrids. Thus, we have previously found that hybrids between NS-1 and human tonsillar lymphocytes stimulated *in vitro* with influenza virus initially secreted human antibody specific for the stimulating virus but failed to retain this capacity through subsequent cloning procedures (8). Nevertheless, long-term human Ig-secreting hybrids like the B1-29 line can occasionally be produced. Stable hybrids that secrete human Ig reactive against the Forssman antigen (16), antigens on human mammary carcinoma cells (17), and human keyhole limpet hemocyanin (18) have recently been reported.

Mouse-human hybrids tend to retain murine chromosomes and lose human chromosomes after fusion (3). Loss of the chromosome bearing the structural genes for human Ig provides one possible explanation for the cessation of Ig secretion. Although disadvantageous with respect to sustained production of human Ig, the preferential loss of human chromosomes from these hybrids provides a useful tool for gene mapping studies. On this basis, Croce et al. (5–7) have suggested that the human heavy chain genes reside on chromosome 14, despite an earlier assignment to chromosome 6 (19, 20).

We have investigated whether the loss of human Ig secretion by our mouse-human hybrids could be attributed to a loss of the chromosome bearing the relevant structural genes. To do this, cells from the secreting B1-29 line were cloned repeatedly to obtain a panel of stable high-secreting and nonsecreting subclones. The majority of nonsecreting subclones proved to have lost surface Ig (Table II). Representative B1-29 subclones and cells from the three original hybrid lines were examined for the presence of chromosomes 6 and 14 using the isoenzyme markers glyoxalase I and nucleoside phosphorylase, which are assigned to these chromosomes (15). This approach proved to be more satisfactory than karyological analysis, because of the very high numbers of chromosomes present in the hybrid cells; B1-29, for example, had a mean of 122 chromosomes. None of the mouse-human hybrids examined expressed human glyoxalase I, implying the absence of chromosome 6; this finding is in agreement with the absence of HLA antigens on the three original hybrid lines, although all of them displayed surface H-2<sup>d</sup> antigens (Table I). E2-42 and all subclones of B1-29 examined, whether secreting or nonsecreting, had human nucleoside phosphorylase, indicating

the presence of human chromosome 14. The remaining line A2-31, which no longer secreted or expressed surface human Ig, lacked human nucleoside phosphorylase. The presence of chromosome 14 and the absence of chromosome 6 in all secreting subclones of B1-29 is in agreement with the assignment of human heavy chain genes to chromosome 14 (5-7) rather than to chromosome 6 (19, 20). On the other hand, we, like Croce et al. (6), observed that many hybrids retained chromosome 14 but did not secrete (Table III). Two possible inferences may be drawn from these findings. Firstly, loss of Ig secretion may be caused by the loss of a regulatory gene rather than a structural gene. Alternatively, but less likely, the chromosomal assignment for human heavy chain genes may be incorrect. To distinguish between these two possibilities, attempts were made to restimulate nonsecreting cell lines to Ig secretion using LPS alone, or PWM with added human T cells. These agents were selected as LPS is a well known polyclonal activator for murine B cells (21) and PWM with T cell help can activate human B cells (22). Furthermore, both of them will stimulate Ig production in malignant as well as normal B cells. For instance, PWM in the presence of T cells has been shown to stimulate peripheral blood mononuclear cells from chronic lymphocytic leukemia patients to secrete Ig (23, 24). Similarly, Gronowicz et al. (25) have induced IgM secretion in a murine B cell tumour with LPS.

Culture of the nonsecreting mouse human hybrids with PWM and different ratios of normal human T cells failed to induce the secretion of human Ig (data not shown). Irradiation of the human T cells in this system, for the purpose of removing suppressor cells (26), also failed to result in human Ig secretion (data not shown). By contrast, treatment with LPS did induce the secretion of human Ig from previously nonsecreting mouse-human lines (Fig. 2). In other words, the hybrids appeared to resemble mouse cells more closely than human cells in their response to mitogens. The level of secretion depended on the number of cells used, as a maximum response was seen with an initial cell concentration of  $1 \times 10^4$  cells/ml, whereas cells cultured at higher concentrations ( $5 \times 10^4$  cells/ml) yielded much lower amounts of Ig. Hybrid cells reacted to LPS by secretion of human IgG at levels of up to  $0.7 \mu\text{g/ml}$ , the greatest response occurring at a concentration of  $10 \mu\text{g/ml}$  LPS. A high response was seen after 1 d in culture, and in two of the three lines examined, this response was maintained over the next 4 d (Fig. 3). Surprisingly A2-31, which lacks human nucleoside phosphorylase, did produce Ig, albeit transiently. The ability to restimulate A2-31, despite the apparent absence of chromosome 14, may have a number of explanations. For example, a translocation may have occurred involving chromosome 14, with subsequent loss of genes for nucleoside phosphorylase and retention of genes for human Ig. Alternatively, the nucleoside phosphorylase gene may not be phenotypically active in A2-31, leading to the false assumption that chromosome 14 was absent. Whatever the explanation (if either is correct), these data illustrate the need for caution in using enzyme markers for gene assignment.

The capacity of LPS to restimulate Ig secretion in nonsecreting mouse-human hybrids demonstrates that loss of secretion is not necessarily due to loss of the structural genes for Ig. Instead, there may be a loss of genes regulating Ig synthesis or secretion, a defect that can be overcome by treatment with the appropriate mitogen. From the practical point of view, mitogen treatment may provide a means of reclaiming Ig secretion in hybrids that have become nonfunctional. This approach may be relevant not only to interspecies hybrids, but also to human-human hybrids,



where loss of secretion in the cloning stages also appears to be a problem (E. Adams and G. McCaughan, personal communication).

### Summary

Three cloned mouse-human lines (B1-29, E2-42, and A2-31) secreting human immunoglobulin (Ig) were obtained from a fusion between the mouse myeloma line NS-1 and human tonsillar lymphocytes stimulated in vitro with pokeweed mitogen. One line, B1-29, has continued to secrete human IgG for a period of 2 yr in culture. This line was recloned three times to give a panel of secreting and nonsecreting subclones. Most of the nonsecreting subclones had also lost surface Ig.

The structural genes for human Ig heavy chains have been provisionally assigned to chromosome 14, which also encodes the enzyme nucleoside phosphorylase. Human nucleoside phosphorylase was detected in all secreting and nonsecreting B1-29 subclones, indicating the presence of human chromosome 14. The retention of chromosome 14 in nonsecreting clones implied that the structural genes for human Ig were still present. Using one of the B1-29 nonsecreting clones and the original cell lines, A2-31 and E2-42, which had stopped secreting, an attempt was made to restimulate the secretion of human Ig with mitogens. A2-31 was unique among the cell lines examined, in that chromosome 14 could not be detected by an isoenzyme marker. Lipopolysaccharide, at an optimum dose of 10  $\mu\text{g/ml}$ , restimulated these nonsecreting hybrid lines to secrete human IgG in levels up to 0.7  $\mu\text{g/ml}$ . Loss of Ig secretion may not therefore be caused by loss of Ig structural genes.

We thank Miss J. Watson for valuable assistance with chromosomal preparations, Miss E. Adams for expert help with the culture of human lymphocytes, and Miss D. Bartimote for careful preparation of the manuscript.

*Received for publication 3 May 1982 and in revised form 14 July 1982.*

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