FUSION AND HYBRIDIZATION OF HUMAN LYMPHOBLAST CELLS

by

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INTRODUCTION

The genetic regulatory mechanisms which govern the expression of differentiated functions in mammalian cells are to a large extent still unknown. However, recent development of techniques for establishment of differentiated cells in tissue culture, for induction of mutant mammalian cells with selectable biochemical markers, and for production of somatic cell hybrids between clones of differentiated and undifferentiated cells has provided a means of studying these genetic regulatory mechanisms *in vitro*.

We have attempted to investigate the means by which immunoglobulin production is regulated in hybrids between cultured human lymphoblast cells and mouse fibroblast cells. Our aims were to isolate clonal sublines of the human lymphoblast cell lines present in our laboratory, to characterize immunoglobulin production in these cloned sublines, to fuse cloned lymphoblast cells with various human, mouse, and hamster fibroblast cells, and to assay immunoglobulin production in the resultant somatic cell hybrids.

Due to a number of procedural problems, stemming mostly from the poor fusion abilities of human lymphoblast cell lines, these aims were only partially accomplished. The majority of our work involved the development of a trypsin-centrifugation

technique which effectively increased lymphoblast x fibroblast fusion and hybridization frequencies. Those experiments are presented in a form suitable for publication in Experimental Cell Research in the first section of the thesis. Remaining data is included in the appendix.

PAPER: VIRAL AND NONVIRAL INDUCTION OF FUSION AND HYBRIDIZATION OF HUMAN LYMPHOID CELLS

SUMMARY

Frequency of fusion and hybridization of human lymphoblasts and fibroblastic cell lines was increased significantly above control levels by simple co-centrifugation of previously trypsinized parental cells. Treatment of cells with Sendai virus also stimulated fusion and hybridization, but the frequencies of hybrids were 11 to 13 times greater with the trypsinization-centrifugation technique. Frequencies of multinucleated cells were lower when cells were not co-centrifuged or when fetal calf serum was added to the cell suspension before centrifugation. Spontaneous and induced frequencies of fusion and hybridization varied in comparisons of four alternate pairs of lymphoblast and fibroblast parental cell lines. Cell fusion enhancement by trypsin depended upon concentrations and activities of enzyme preparations.

Inactivated Sendai virus has proved useful in promoting cell-cell fusion and increasing mammalian somatic cell hybrid frequencies. Okada and Tadokoro (1), however, noted that the efficiency of Sendai-mediated cell fusion varied, and that mouse, human, and rabbit leukocytes did not appear to fuse after Sendai treatment. The relative resistance of human leukocytes to Sendai-mediated cell fusion was recently substantiated with primary tonsil lymphocytes (2) as well as with long-term, established lymphoid cell cultures (3,4).

Previous work with cell lines resistant to fusion suggested a number of factors that may alter cell fusion frequencies; e.g., mating ratio of parental cells (5), cell cycle stage of parental cells (6-8), thickness of cell surface coating (9), cholesterol: phospholipid ratio of the cell membrane (10), and pH of the culture medium during and after cell fusion (11,12). Nonviral agents like lysolecithin (13,14) also may increase cell fusion, thus avoiding the necessity of using a viral agent which could retain biological function (15).

Stadler and Adelberg (7) recently reported that the frequency of Sendai-induced fusion of interphase mouse leukemic cells was lower than that of cells in mitosis. They observed, however, that a 10 min treatment of interphase cells with 0.01% trypsin before exposure to Sendai virus significantly increased cell fusion. Sun et al. (16) and Bloom and Nakamura (17) successfully used the Stadler-Adelberg observation to increase frequencies of Sendai-induced hybridization of human Burkitt's lymphoma cells and established lymphocyte cultures.

The studies reported here indicate that cell fusion and hybridization of established human lymphoid cells in culture can be significantly stimulated by a trypsin-centrifugation procedure that requires no Sendai virus. In addition, our observations suggest that various lymphoid cell lines differ in fusion and hybridization capabilities and that Sendai virus may promote fusion of some cell lines without increasing hybrid frequencies.

MATERIALS AND METHODS

Cell Lines

Established cultures of human lymphoblast cells, initiated from peripheral blood samples of patients at the Massachusetts General Hospital, were kindly provided by Dr. J.W. Littlefield (John Hopkins Medical School). The MGL5 cell line was initiated from an 18-year-old female with infectious mononucleosis; the MGL7 line, from a 14-year-old female with a minor foot infection, and the MGL8 line, from a 27-year-old healthy male. The MGK8E culture was a once-cloned subline of MGL8 isolated as a single colony after plating in soft-agarose medium, as described previously (18). The T-5-1 lymphoblast cell line was an hypoxanthine-guanine phosphoribosyl transferase (HGPRT) enzyme-deficient mutant previously isolated (18). TG2 ($HGPRT^-$) hamster cells, B1 thymidine kinase (TK)-deficient hamster cells, B82 (TK^-) mouse cells, and LN326 ($HGPRT^-$) human fibroblasts from a patient with the Lesch-Nyhan syndrome were also provided by Dr. J.W. Littlefield. The 3T3c2F (TK^-) mouse cell line was provided by Dr. C. Basilico (New York University Medical Center). RAG (TK^-) mouse cells were obtained from the American Type Culture Collection. Various cell lines grown in our laboratory were periodically checked for *Mycoplasma* contamination by Dr. L. Hayflick (Stanford University) and were consistently negative.

Maintenance of Cell Cultures

Mouse, hamster, and human fibroblasts were maintained in

Eagle's minimum essential medium (MEM) (19) supplemented with 11% heat-inactivated fetal calf serum (FCS), 1.0 g/l sodium pyruvate, 0.2 mg/l $Fe(NO_3)_3 \cdot 9H_2O$, and nonessential amino acids. Lymphoblast cells were maintained as previously described (18), except that the R.P.M.I. 1640 growth medium (20) was supplemented with 15% heat-inactivated FCS. Serum and powdered media were purchased from Grand Island Biological Co. Lymphoblast cells were passed regularly into fresh medium at 8- to 12-day intervals and only actively growing cultures in the logarithmic stage of growth were used for cell fusion and hybridization experiments. Fibroblast cell lines were routinely dispersed for experiments and transfer by rinsing (1X) with Dulbecco's phosphate buffered saline (PBS) and incubating for 5-10 min with 1.0 ml of 0.25% Bacto-trypsin per 100 mm culture dish. Cell numbers were determined with a model Z Coulter counter, which was standardized regularly with a pre-counted latex-bead suspension (Clinton Laboratories).

Sendai Virus Induced Fusion

Sendai virus, originally obtained from Dr. B. Knowles (Wistar Institute), was grown and inactivated with 0.025% β-propiolactone, as described by Smith and Consigli (15). The amount of virus was quantitated by hemagglutination assays using guinea pig red blood cells washed in Alsever's solution. Completeness of viral inactivation was checked by inoculating 10-day-old fertile eggs with 1×10^5 hemagglutinating units (HAU) of β-propiolactone-

treated virus; increases in HAU titers were not observed after 6 days' incubation. Inactivated virus was stored frozen at -93° C and rapidly thawed at 37° C before use.

Lymphoblast:fibroblast cell ratios were varied from 1:1 to 1:10 for fusion experiments, a total of 1×10^7 cells in 1.0 ml. Cells were co-centrifuged for 10 min at 500 xg, rinsed once with 10 mls of Hanks balanced salt solution (HBSS), and resuspended in 1.0 ml of Sendai virus at 4° C. Cells were incubated with virus for 20 min on ice, maintained at 37° C for 20 min, diluted with 9 mls of HBSS, and centrifuged at 500 xg to remove unadsorbed virus. Cell pellets were resuspended in culture medium and plated at $1\text{-}2 \times 10^5$ cells per 100 mm Falcon culture dish in HAT medium (21) for hybrid selection, or plated at $1\text{-}2 \times 10^4$ cells in 30 mm Falcon dishes, each containing a sterile glass coverslip, for determining number of nuclei/cell.

Determination of Frequencies of Multinucleated Cells and Somatic Cell Hybrids

Hybrids were selected by rinsing off the lymphoblast parental cells (which grow in suspension) with PBS at 2-3 day intervals and refeeding the remaining fibroblastic cells with fresh MEM-HAT medium. Hybrids of enzyme-deficient fibroblast cells and lymphoblasts were selected by growth of attached colonies in HAT medium. Hybrid colonies grew with a fibroblastic morphology and were macroscopically evident at 3-4 weeks after plating. Hybrid frequency calculations were based on the number of fibroblastic

parental cells plated. Reversion frequencies of enzyme-deficient fibroblast mutants 3T3c2 (TK^-), B82 (TK^-), LN326 ($HGPRT^-$), or TG2 ($HGPRT^-$) to the wild type were lower than 1×10^{-7}; no revertants capable of growth in HAT medium were observed.

Cell fusion was quantitated by counting the fibroblastic cells with multiple nuclei at 20-24 hours after plating. Cells growing on glass coverslips were fixed for 10-15 min in PBS containing 3.7% formaldehyde, stained with Leischman's stain (Gurr's 1X), and microscopically examined at 400X with bright-field or Nomarski optics. Approximately 200-300 cells were counted per coverslip and 3-5 coverslips were examined for each treatment.

Trypsin Treatment

Bacto-trypsin (Difco), purchased as a lyophilized powder in sterile vials, was diluted to concentrations of 100-5000 ug/ml in PBS. Twice crystallized trypsin (Worthington) was obtained in a salt-free and lyophilized form (specific activity 195 units/mg) and was diluted to concentrations of 0.1 to 50 ug/ml in PBS. Bacto-trypsin had 0.7% of the tryptic esterase activity of the crystalline trypsin on p-toluenesulfonyl-arginine methyl ester (TAME) substrate.

Parental cells (1×10^7 cells/ml) were mixed for cell fusion as described for Sendai experiments, co-centrifuged for 10 min at 500 xg, and rinsed once in HBSS. Cell pellets were resuspended in 1.0 ml of trypsin, incubated in a 37° C water bath for 10 min,

diluted with 4-5 mls of HBSS, and then centrifuged for 10 min at 500 xg to separate cells from enzyme. Pelleted cells were resuspended in MEM and plated as described for Sendai experiments.

Lysolecithin-Induced Fusion

A total of 5-10 x 10^6 parental cells were mixed, co-centrifuged, and rinsed in HBSS as previously described. The cell pellets were resuspended in 0.1 ml of lysolecithin solution (Supelco), which contained 400 ug/ml of lysolecithin and 5 mg/ml bovine serum albumin (13) in PBS (pH 7.2). Cells were treated with lysolecithin for one minute (22° C), diluted 1:10 with MEM (30% FCS), and plated as described above.

RESULTS

Effect of Sendai Virus on Lymphoblast Fusion and Hybridization

Sendai virus did not stimulate hybridization of MGL5, MGL7, or MGL8 diploid lymphoblast cells with mouse or hamster fibroblasts. Initial experiments used 500-5000 HAU of Sendai virus and 5 x 10^6 each of lymphoblast and fibroblast parental cells. The negative result was surprising, as similar treatments of hamster (B1 x TG2) fibroblasts with 0, 500, 1000, or 5000 HAU of the same viral preparation increased hybrid frequencies from 4.4 x 10^{-3} (spontaneous) to 10.8, 11.0, and 23.0 x 10^{-3}, respectively. In addition, in the latter experiments, generalized clumping and adherence of lymphoblasts to fibroblasts was markedly stimulated by Sendai virus.

We attempted to increase lymphoblast hybrid frequency in subsequent experiments by controlling medium pH (11) during and

after cell fusion. Equal numbers of MGK8E lymphoblasts and 3T3c2 (TK^-) fibroblasts (5-10 x 10^6/ml) were treated with or without Sendai virus in HBSS buffered at pHs 6.8-8.0 with 20 mM HEPES-5 mM sodium bicarbonate buffer. Percentages of multinucleated cells induced by Sendai treatment were slightly higher (1.4-1.5 fold) than controls at pH 7.4 and 7.6 (Table 1), but again, no hybrid colonies were obtained.

Varying parental cell ratio (17) was also important in stimulating hybridization of lymphoblasts. Preliminary experiments indicated that fusion of parental cells in ratios of 2:1 to 10:1 MGL8 lymphoblasts:rodent fibroblasts (or the converse) resulted in maximum hybridization when ratios of lymphoblasts:fibroblasts were high. Maximum frequencies of hybrids were 2-3 x 10^{-6} at ratios of 2:1 lymphoblasts:fibroblasts (representative data in Table 3) and were not increased at higher ratios. The relatively low hybridization obtained with Sendai virus prompted us to investigate nonviral methods of increasing cell fusion and hybridization.

Effect of Trypsin on Lymphoblast Fusion and Hybridization

Preliminary experiments, in which lymphoblasts were treated with trypsin before Sendai virus was added, were performed as suggested by Stadler and Adelberg (7). We observed that fusion of lymphoblasts was enhanced by trypsin alone, and frequencies of multinucleated cells were not further increased by addition of Sendai virus. This suggested that treating parental cells with

trypsin might increase hybridization of lymphoblasts without Sendai virus. The comparative abilities of Sendai virus or trypsin to induce cell fusion and lymphoblasts and various rodent or human cell lines were investigated in experiments summarized in Table 2. Table 2A shows frequencies of multinucleated cells obtained with Sendai virus or trypsin from four lymphoblast cell lines and mouse 3T3c2 (TK^-) cells. Slightly more multinucleated cells were induced by Sendai virus than by trypsin for each lymphoblast line tested. However, though the data showed that different lymphoblast cell lines varied in fusion capability, trypsin and Sendai virus did not differ significantly in inducing cell fusion. The MGK8E x 3T3c2 combination of parental cells was the only example in which fusion was similarly enhanced by Sendai virus or trypsin. The MGL7 x 3T3c2 parental cells, in contrast, had the highest spontaneous frequencies of cell fusion and these frequencies were reduced following trypsin treatment.

Fusion of MGK8E lymphoblasts with different human or rodent fibroblast cell lines (Table 2B) again indicated that cell fusion was increased similarly by either trypsin or Sendai virus, as observed with various lymphoblasts and 3T3c2 cells (Table 2A). Highest frequencies of multinucleated cells were observed with the TG2 x MGK8E or with RAG x MGK8E parental cells; lowest frequencies with either LN326 or B82 x MGK8E parental cells. Trypsin appeared to consistently stimulate multinucleated cell formation as well as did Sendai virus.

In our next studies we attempted to determine whether the observed multinucleated cells resulted from the experimental treatments or, because of high spontaneous frequencies, were already present in the various fibroblastic cell populations. The incidence of multinucleated cells in untreated fibroblasts varied from 0.08-0.10% for most of the rodent cell lines (RAG, B1, B82, and 3T3c2), but was higher for the LN326 human fibroblasts (1.0%) and the TG2 hamster cells (2.6%). Data in the tables were not corrected for those relatively low spontaneous values. Rather, each experiment was compared to control cells, which were mixed and co-centrifuged but not treated with trypsin or Sendai virus. That method proved to be more realistic than comparisons with spontaneous frequencies of homokaryotic cells because mixing and co-centrifugation of parental cells apparently increased multinucleated cell number (Table 2, control values) above the spontaneous.

We also studied the incidence of homospecific cell fusion by examining frequencies of multinucleated cells produced after treatment of 3T3c2 x 3T3c2 cells with Sendai virus or trypsin. Multinucleated cells increased 1.9 fold (5.7%) with 2500 HAU of Sendai virus and 2.3 fold (4.9%) with trypsin. Those results support the possible contribution of homospecific cell fusion to the multinucleated cell increase (Tables 1 and 2). However, since the marked increase in the incidence of hybrid colonies

following certain treatments in later experiments (Tables 3 and 4) indicated induction of heterospecific fusion, we did not attempt to specifically identify or quantitate the homo- or heterokaryotic cells in that portion of the study. Our primary goal was to increase the frequency of cell hybrids.

Induction of Cell Hybridization

Table 3 compares hybridization frequencies for lymphoblasts and 3T3c2 (TK^-) fibroblasts mixed in a 2:1 ratio and treated with Sendai virus or trypsin. Treatment with Sendai virus, previously shown to increase frequencies of multinucleated cells more than trypsin (Table 2), increased the hybridization frequency of MGK8E x 3T3c2 (TK^-) cells to only 3.3×10^{-6}, a 1.39 fold increase over the control values (Table 3A). Trypsin treatment, in contrast, markedly increased the hybridization frequency of MGK8E x 3T3c2 cells to 42.6×10^{-6}, or 12.9 times the maximum hybridization frequencies obtained in the Sendai experiments. However, neither Sendai virus nor trypsin enhanced hybridization of T-5-1 x 3T3c2 parental cells (Table 3B). Interestingly, control hybridization frequencies for those particular parental cells were higher than those of cells treated with either fusion agent, and significantly higher than other lymphoblast x 3T3c2 combinations.

Hybridization frequencies of control (untreated) cells in the trypsin experiments (2.7×10^{-5}) were 11 times higher than those of control cells in the Sendai experiments (0.24×10^{-5}), as shown

in Table 3. Some factor inherent in the trypsin experimental procedure apparently was principally responsible for increasing lymphoblast hybridization. Earlier experiments, during preliminary studies on trypsin-induced cell fusion and hybridization, indicated that co-centrifugation of parental cells following enzyme treatment was required to increase frequencies of cell hybridization; few, if any, hybrids appeared when the centrifugation step was omitted. Data in Table 4 are consistent with that observation. Co-centrifugation of parental cells, which were mixed together but not treated with trypsin, resulted in more multinucleated cells (Table 4) than were found in non-centrifuged spontaneous (homokaryotic) cell populations. Frequencies of cell fusion (Table 4) or hybridization (Table 3A) were higher, however, when parental cells were treated with trypsin before co-centrifugation. We observed a 1.93 fold increase in multinucleated cell number when trypsinized parental cells were centrifuged (Table 4B), a greater increase than that of trypsinized, but non-centrifuged, cells (Table 4C). Multinucleated cells were fewest when medium containing 30% FCS was added to the cells before centrifugation (Table 4B), or when trypsin was omitted (Table 4C). Those data, nevertheless, are still insufficient to explain why hybridization frequencies for control cells in Sendai experiments were low, because they also were co-centrifuged after incubation with virus.

Effect of Trypsin Concentration

Trypsin enhancement of cell hybridization frequencies depended

on the purity and concentration of the enzyme. Maximum frequencies ($5.4\text{-}5.5 \times 10^{-5}$) followed a 10 min treatment with either 2500 ug/ml Bacto-trypsin or 50 ug/ml crystalline trypsin (Table 5). Hybridization frequencies of MGK8E x 3T3c2 parental cells were 18 times higher than the maximum previously obtained with Sendai virus (Table 3A). Hybridization frequencies generally increased after treatment with higher concentrations of either enzyme preparation. Treating cells 20 min with trypsin (not shown) or with concentrations of Bacto-trypsin exceeding 2500 ug/ml lowered hybrid frequencies. Treating cells with 5000 ug/ml Bacto-trypsin consistently decreased hybridization, but the highest concentration of crystalline trypsin used did not. Bacto-trypsin had 0.7% the activity of Worthington trypsin (Materials and Methods), suggesting that even higher concentrations or longer exposure periods may more effectively increase hybrid frequencies with crystalline trypsin, and/or that Bacto-trypsin at higher concentrations may contain a contaminant toxic to cells.

Alternative Nonviral Methods for Lymphoblast Fusion

We found lysolecithin ineffective for stimulating lymphoblast fusion or hybridization, and observed extensive cell lysis and failure of cells to re-attach to the culture dish after treatment of MGK8E x 3T3c2 cells with 400 ug/ml lysolecithin. Recent experiments with lower concentrations of lysolecithin (5-100 ug/ml) indicated that mouse cells survived and re-attached to the culture dish after treatment, but hybrid frequencies were not increased.

Treatment (10 min at 37° C) of parental cells with hyaluronidase (Type III, 435 units/mg; Sigma) or neuraminidase (Type V, 0.12 units/mg; Sigma) before co-centrifugation was less effective than trypsin treatment for increasing frequencies of cell fusion (Table 6). In summary, frequencies of multinucleated cells were not increased after treating either MGK8E x 3T3c2 or MGK8E x LN326 parental cells over a range of 0.10-100 units/ml of hyaluronidase. Neuraminidase did not increase frequencies of multinucleated cells with the MGK8E x 3T3c2 parental cells, but slightly stimulated fusion (1.6 fold) over controls with the MGK8E x LN326 parental cells at 0.1 unit/ml, the highest concentration used. Additional experiments with increased enzyme concentrations, with assured (known) purity, and with varied times of treatment are necessary, especially with neuraminidase, to confirm preliminary data presented here and to determine whether or not hybridization frequencies also are increased.

DISCUSSION

Fusion and hybridization of diploid human lymphoblasts were stimulated in the absence of Sendai virus by treating mixed parental cells with trypsin, followed by low-speed centrifugation. Frequencies of MGK8E x 3T3c2 hybrid colonies were markedly lower for cells fused with Sendai virus (3.0×10^{-6}) than for cells fused with trypsin and centrifugation ($40\text{-}50 \times 10^{-6}$). The relative ineffectiveness of Sendai virus for stimulating hybridization of human lymphoblasts is consistent with other reports on human leukocytes (2,3).

Frequencies of multinucleated cells in our studies were lower when parental cells were not treated with trypsin or when fetal calf serum was added to the trypsin-treated cells before centrifugation.

Co-centrifugation of lymphoblast and fibroblast cells (without trypsin) increased hybrid colonies 9-11 fold over control levels in Sendai virus experiments, a result difficult to explain because control cells in the Sendai virus experiments also were co-centrifuged, but still had low hybridization frequencies (Table 3A). A possible explanation is that control cells were cooled on ice 20 min in Sendai experiments, but were not cooled in trypsin studies. Lin et al. (26) observed that chilled lymphocytes had fewer microvilli on cell surfaces than control cells incubated at 37^{o} C. Poste (9) suggested that microvilli may effectively increase cell surface area and, thus, increase the likelihood of cell-cell attachments. However, our results indicated that average frequencies of cell fusion of Sendai control cells (which were chilled) were nearly identical to trypsin control cells (which were not chilled), a finding which suggests that the difference in hybridization frequencies for the respective control cells results from a post-fusion event. Unfortunately, we do not now have enough data for meaningful comments on mechanism(s) that may inhibit hybridization of heterokaryotic cells. Such data might become available from experiments designed to determine the number of heterokaryotic cells remaining in the population as a function of time after each treatment or to determine whether or not temperature changes inhibit hybrid formation.

It seems reasonable to assume that trypsin and centrifugation treatments of lymphoblasts and fibroblasts may change cell surfaces and, thus, render parental cells more conducive to cell-cell interactions (9). The action of trypsin at the cell surface seems to be well established. Trypsin treatment of BHK cells caused significant losses of cell surface proteins, polysaccharides, and sialic acid residues (27). Removal of sialic acid from chick fibroblasts stimulated cell-cell adhesion and aggregation (28). Trypsin or neuraminidase treatment of lymphoid or fibroblastic cells also 1)changed the surface charge on the cell (29); 2) exposed lectin binding sites on the cell surface (30); 3) altered the electrophoretic mobility of the cell (31,32); and 4) increased the immunogenicity of injected lymphocytes (33). Thus, altered cell surface proteins, structure, or electrostatic charge may be involved in increasing lymphoblast-fibroblast interactions that occur rarely between untreated cells. Neuraminidase, but not hyaluronidase, treatment of parental cells (followed by co-centrifugation) produced a moderate but significant ($P < 0.05$) increase in the frequencies of multinucleated human lymphoblasts and fibroblasts at high enzyme concentrations. In contrast, similar increases in cell fusion were not observed with lymphoblasts and 3T3c2 mouse cells. Our data, however, are only preliminary indications of the usefulness of neuraminidase; longer treatment times or higher enzyme concentrations may be required for maximum stimulation of fusion.

Co-centrifugation of cells also may alter cell surface properties. Milam and Srere (34) observed that more BHK cells attached to polystyrene beads after centrifugation and that addition of serum to the cells before centrifugation inhibited attachment. We do not yet know whether fetal calf serum simply inactivates trypsin or lowers the frequency of fusion via a component that interacts with the cells. However, we know that fetal calf serum contains hyaluronic acid (34), and that hyaluronic acid inhibits fusion of protozoan cells (Echinosphaerum sp.) (36). Further studies with purified hyaluronic acid and soybean trypsin inhibitor are necessary to distinguish between those proposed roles of serum.

Fusion potential variation among lymphoblast lines was most obvious in our study with the T-5-1 and MGL7 cell lines. T-5-1 and MGL7 had high spontaneous frequencies of fusion in the absence of Sendai or trypsin-centrifugation; neither cell line fused after either treatment. Orkin et al. observed high spontaneous hybridization frequencies for T-5-1 (3) which may be related to: the hypodiploid chromosome number of the cells (37); the extended time (7-8 years) that this clone (or its parental line) has been grown in continuous culture (38); or, a possible alteration in cell surface properties after induction of the HGPRT-deficient mutant subline with ethylmethane sulfonate (18).

Further investigation of the molecular mechanisms for variability in lymphoblast fusion and hybridization is needed. Our current

data suggest that trypsin treatment and co-centrifugation of lymphoblast cells may serve as a rapid and alternative nonviral method for stimulating frequencies of somatic cell fusion and hybridization. The procedure also may prove useful for other poorly fusing lymphoid cells.

Table 1. Percentages of multinucleated cells induced by Sendai virus with MGK8E lymphoblasts and 3T3c2 (TK^-) mouse fibroblasts treated at indicated pH levels.

pH during and after treatment	Mean percentage multinucleated cells[a] ± S.E.		
	0 HAU[b]	2500 HAU	Ratio Sendai/None
6.8	3.50 ± 0.77	3.71 ± 0.34	1.06
7.0	3.20 ± 0.51	3.50 ± 0.43	1.09
7.2	2.90 ± 0.35	3.56 ± 0.34	1.23
7.4	2.96 ± 0.60	4.56 ± 0.52	1.54[c]
7.6	3.34 ± 0.27	4.81 ± 0.55	1.44[c]
7.8	3.83 ± 0.64	4.72 ± 0.56	1.23
8.0	3.93 ± 0.15	4.74 ± 0.23	1.21

[a] Results of 2 experiments; a minimum of 600 cells was counted in each experiment.

[b] Control cells (without Sendai virus) and treated cells were centrifuged and incubated identically.

[c] $P = 0.05$-0.025 (T-test); other increases not significant at 5% level.

Table 2. Percentages of multinucleated cells induced by treating indicated pairs of lymphoblast and fibroblast cells with Sendai virus or trypsin.[a]

Parental cell lines	Sendai treatment: Percentage of multinucleated cells ± S.E. 0 HAU	Sendai treatment: Percentage of multinucleated cells ± S.E. 2500 HAU	Ratio[b] Sendai/None	Trypsin treatment: Percentage of multinucleated cells ± S.E. 0	Trypsin treatment: Percentage of multinucleated cells ± S.E. 0.25%	Ratio[b] Trypsin/None
A. MGK8E x 3T3c2	3.8 ± 0.53	7.7 ± 1.5	2.0	3.61 ± 0.65	6.9 ± 1.3	1.9
MGL7 x 3T3c2	8.2 ± 1.43	10.4 ± 0.23	1.3	11.2 ± 3.4	9.8 ± 0.72	0.9
MGL5 x 3T3c2	2.6 ± 0.29	4.1 ± 0.37	1.6	2.8 ± 0.29	2.8 ± 0.51	1.0
T-5-1 x 3T3c2	6.1 ± 1.62	8.7 ± 2.8	1.4	6.6 ± 1.9	8.1 ± 2.5	1.2
B. TG2 x MGK8E	6.2 ± 0.67	9.9 ± 0.22	1.6	6.7 ± 0.44	13.9 ± 1.29	2.1
RAG x MGK8E	6.4 ± 0.53	10.1 ± 0.51	1.6	4.2 ± 0.79	9.8 ± 0.55	2.3
LN326 x MGK8E	3.3 ± 0.42	5.6 ± 0.21	1.7	2.5 ± 0.42	5.0 ± 0.29	2.0
B82 x MGK8E	3.2 ± 0.49	5.4 ± 0.95	1.7	2.5 ± 0.25	4.0 ± 0.28	1.6

[a]Data represent averages of at least two experiments and examination of at least 4000 cells.

[b]Differences between ratios of the Sendai and trypsin experiments were not significant ($P < 0.05$).

Table 3. Frequencies of human lymphoblast x mouse fibroblast hybrid cells induced by treatments with Sendai virus or trypsin.

Cell lines	Treatment	Concentration	Total number[a] of cells plated ($\times 10^5$)	Number of hybrid colonies	Hybrid frequencies[b] ($\times 10^{-5}$)	Ratio Treated/ Control
A. MGK8E x 3T3c2	Sendai	0	38	9	0.24	-
		2500 HAU	33	10	0.30	1.25
	Bacto-trypsin	0	28	76	2.7	-
		2500 ug/ml	34	145	4.3	1.59[c]
B. T-5-1 x 3T3c2	Sendai	0	5.9	185	31.3	-
		2500 HAU	6.4	130	20.3	0.65[c]
	Bacto-trypsin	0	12.2	21	1.7	-
		2500 ug/ml	12.2	20	1.6	0.94

[a] Cells plated at 5-10 x 10^4 cells/60-mm dish in MEM-HAT medium.

[b] $P(\text{treatments}) < 0.01$.

[c] $P = 0.1\text{-}0.05$ (T-test); other increases did not differ significantly from controls.

Table 4. Percentages of multinucleated cells produced by indicated alternate treatments of MGK8E lymphoblasts and 3T3c2 (TK^-) fibroblasts.

Method of fusion	Mean percentage of multinucleated cells[a] ± S.E.	Ratio Treated/Control
1. Experiment A		
0 HAU Sendai virus	3.59 ± 0.63	-
2500 HAU Sendai virus	6.29 ± 0.89	1.75[b]
2. Experiment B (cells co-centrifuged)		
0 ug/ml Bacto-trypsin	3.11 ± 0.55	-
2500 ug/ml Bacto-trypsin	5.99 ± 0.55	1.93[b]
2500 ug/ml Bacto-trypsin + 30% FCS	3.49 ± 0.29	1.12
3. Experiment C (cells not co-centrifuged)		
0 ug/ml Bacto-trypsin	2.81 ± 0.21	-
2500 ug/ml Bacto-trypsin[c]	4.77 ± 0.28	1.70[b]

[a]Average of two experiments.

[b]$P < 0.05$ (T-test).

[c]Cells in trypsin solution were plated directly in culture medium without rinsing.

Table 5. Hybridization frequencies of MGK8E lymphoblasts and 3T3c2 (TK^-) fibroblasts after treatment with indicated trypsin concentrations.

Concentration and source of trypsin	Total cells[a] plated (x 10^5)	Number of hybrid colonies	Hybrid frequencies (x 10^{-5})	Ratio Treated/ None
A. Bacto-trypsin[b] (ug/ml)				
0	20	46	2.3	-
100	8	27	3.4	1.48
500	8	32	4.0	1.74
1000	8	35	4.4	1.91
2500	8	44	5.5	2.39
5000	8	29	3.6	1.56
B. Crystallized trypsin[b] (ug/ml)				
0	20	46	2.3	-
0.1	12	37	3.1	1.35
1.0	12	38	3.2	1.39
5.0	12	20	1.7	0.74
10.0	12	43	3.6	1.56
50.0	12	65	5.4	2.35

[a]Cells plated at 5-10 x 10^4/60-mm culture dish in MEM-HAT medium.

[b]Activity and source in Materials and Methods.

Table 6. Percentages of multinucleated cells induced by hyaluronidase or neuraminidase treatment of lymphoblast and fibroblast cells.

Treatment and cell lines	Enzyme concentration[a] (units/ml)	Mean percentage multinucleated cells[b] ± S.E.	Ratio Treated/ None
A. Hyaluronidase[b]			
MGK8E x 3T3c2	0	2.85 ± 0.76	-
	0.1	3.67 ± 0.08	1.29
	1.0	3.75 ± 0.28	1.32
	10.0	3.08 ± 0.20	1.08
	100.0	3.06 ± 0.50	1.07
MGK8E x LN326	0	2.90 ± 0.21	-
	0.1	2.45 ± 0.43	0.84
	1.0	2.85 ± 0.20	0.98
	10.0	2.80 ± 0.34	0.97
	100.0	2.94 ± 0.40	1.01
B. Neuraminidase[b]			
MGK8E x 3T3c2	0	3.11 ± 0.20	-
	0.00010	2.87 ± 0.14	0.92
	0.0010	2.74 ± 0.34	0.88
	0.010	3.11 ± 0.24	1.00
	0.10	3.34 ± 0.03	1.07
MGK8E x LN326	0	2.18 ± 0.27	-
	0.00010	2.70 ± 0.39	1.24
	0.0010	2.71 ± 0.15	1.24
	0.010	3.20 ± 0.31	1.47[c]
	0.10	3.37 ± 0.43	1.55[c]

[a]Source and activity given in Results.

[b]Minimum of 600 cells examined in each of two experiments.

[c]P = 0.05-0.025 (T-test); other ratios not significant at 5% level.

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APPENDIX

LITERATURE REVIEW

Since Okada (1) first discovered that Ehrlich ascites tumor cells could be induced to form syncytia following treatment with HVJ (Hemagglutinating Virus of Japan or Sendai virus), somatic cell hybridization has become an increasingly important tool in the study of genetic and biochemical regulation in animal cells. Okada's observation led to the idea that virus-induced inclusion of two separate differentiated nuclei in a single cytoplasm might allow the determination of the control mechanisms for differentiated functions.

In 1965, Harris and Watkins (2) successfully used Sendai virus to fuse human and mouse parental cells. A small percentage of these heterokaryotic cells subsequently formed mononuclear hybrids. Nuclear fusion in the heterokaryons occurred in inverse proportion to initial virus concentration, possibly because at higher virus concentrations increasing amounts of plasma membrane damage reduced over-all cell viability. The mononuclear cells resulting from nuclear fusions were designated synkaryons and survived for periods up to two weeks, but formation of colonies was not observed (2,3).

Eventually, Littlefield (4) and Yerganian and Nell (5) isolated <u>viable</u> hybrids (those capable of giving rise to colonies) from, respectively, fusions involving two mouse cell lines and two hamster cell lines. Coon and Weiss (6) successfully produced

viable interspecific hybrids using two mouse L cell sublines and a cloned rat liver cell line. Their experiments indicated that ultraviolet-inactivated Sendai virus served to increase hybridization 100 fold over the spontaneous frequency. Hybrid colony frequency obtained, however, showed little change over a wide range of virus concentrations. This implied that, although large numbers of heterokaryons were formed in the initial fusion process, few went on to produce viable mononuclear hybrids.

The development of the HAT (hypoxanthine-aminopterin-thymidine) selective system for cells with complementary drug-resistant markers by Littlefield (4) permitted detection of very small numbers of hybrid colonies from parental cell populations with poor fusion and hybridization capabilities. The selection technique originally employed two cloned sublines of mouse L cells, one $HGPRT^-$ and one TK^-, selected by virtue of their resistance to 3 ug/ml of 8-azaguanine or to 30 ug/ml of 5-BrdU respectively. In HAT medium, endogenous biosynthesis of purines and thymidylic acid is blocked by aminopterin, a specific inhibitor of folic acid reductase, which catalyzes the synthesis of reduced folate, a compound required at several steps of the purine and pyrimidine biosynthetic pathways. Aminopterin also inhibits glycine biosynthesis, important in purine and pyrimidine biosynthesis. (See Figure 1.) For hybrid selection, neither enzyme-deficient parent cell line can survive due to their respective inability to utilize either the hypoxanthine or thymidine present in the medium, although complementation of these deficiencies occurs in

Figure 1. Endogenous and exogenous purine and pyrimidine biosynthetic pathways (29).

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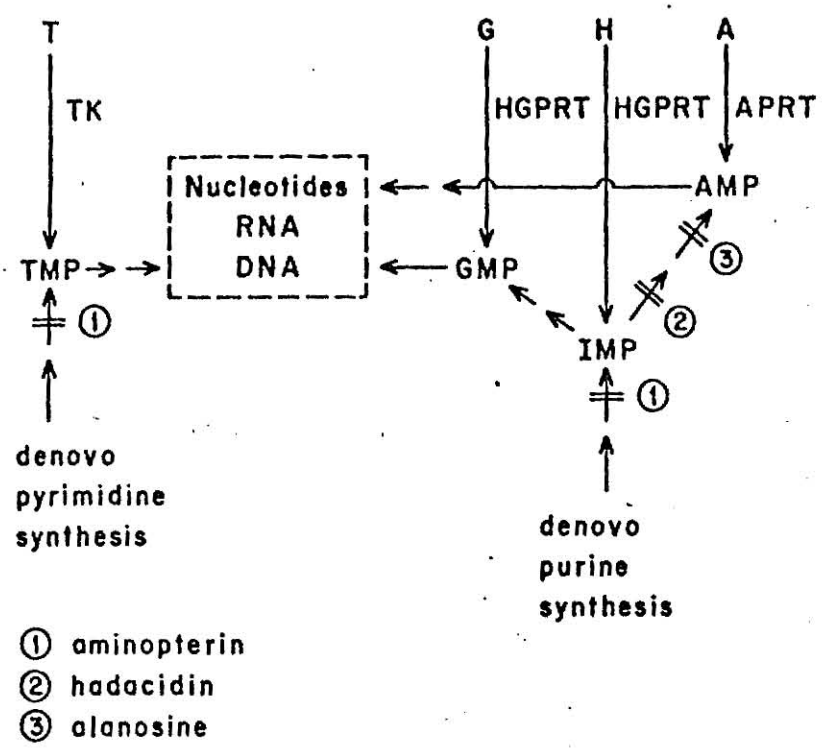
FIGURE 1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② hadacidin
③ alanosine

the hybrids permitting survival. Littlefield's HAT selective system allows for the detection of hybrid cells at frequencies as low as 1-5 x 10^{-6}. Recently, a selective system has been devised by which adenine phosphoribosyl transferase (APRT) deficient mouse x normal human fibroblast hybrids can be detected in low frequencies; the fused cells are grown in a medium containing alanosine, an antibiotic which blocks endogenous synthesis of adenylic acid (7).

There are currently available, however, only a limited number of human cells with suitable biochemical markers for specific hybrid selection. Many of these mutant cell lines are derived from rare patients suffering from various metabolic deficiency diseases such as the Lesch-Nyhan syndrome ($HGPRT^-$), non-spherocytic anemia ($G6PD^-$), citrullinemia, galactosemia, and mucopolysaccharidosis (8-10). Induction of drug-resistant, enzyme-deficient cells by mutagenesis employing X-irradiation or chemical mutagens such as ethyl methane sulfonate (EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), has only recently been successful for human cells. Albertini and DeMars (11) induced 8-azaguanine resistance in diploid human fibroblast cell strains using X-rays; Mankovitz et al. (12) developed ouabain-resistant mutants of diploid human fibroblasts cultured from skin biopsies and of HeLa cells using EMS. Hampar et al. (13) isolated a 5-BrdU-resistant line of Epstein-Barr virus positive Burkitt lymphoma cells. Sato, Slesinski, and Littlefield (14) induced 6-thioguanine-resistant lymphoblasts with EMS and MNNG,

including the T-5-1 cell line used in several of our experiments.

Since the number of biochemical mutants is limited, various workers attempted to devise alternative selective systems for hybridization studies. Davidson and Ephrussi (15) devised a "half-selective" system utilizing a slow-growing normal mouse diploid fibroblast in lieu of one drug resistant parent to circumvent the necessity of using two biochemically marked, enzyme-deficient mutants. Nabholz, Miggiano, and Bodmer (16) utilized non-stimulated human leucocytes in place of a marked cell, thus allowing discrimination between non-dividing leucocytes and hybrids capable of division in selective media.

Unfortunately, the problem of low fusion and hybridization frequencies persists in experiments with human diploid cells. Although there are large numbers of cell types that continue to display differentiated functions when cultured in vitro, many cells, including human leucocytes, have very low fusion and hybridization frequencies, thus limiting their usefulness in the study of the control mechanisms of differentiated capabilities. Okada and Tadokoro (17) compared the abilities of different cell types to fuse upon exposure to Sendai virus and found that established cell lines, mouse ascites tumor cells, and human leukemia cells had high fusion capacities, mouse and chick embryo fibroblasts had low fusion capacities, while human, rabbit, and mouse leucocytes could not be induced to fuse at all.

In attempts to explain and alleviate this problem, cell fusion

capacity variations have been related to a large number of factors. Fusion capacity was most directly related to virus concentration, since polykaryocytosis occurred in proportion to the amount of virus added to the original populations of mononuclear cells (18). Virus type may also influence the ability of a given cell population to fuse and there are many different viruses which will induce cell fusion, including measles, varicella, herpes-, and myxoviruses (19). The single-stranded RNA virus known as Sendai virus or Parainfluenza I is included in this last group and is the most widely used viral fusion agent for hybridization studies.

Stadler and Adelberg (20) found that the Sendai virus-induced fusion capacity of mouse leukemic cells is minimal during the G1 phase of the growth cycle and maximal in colcemid-arrested mitotic cells. Treatment of non-synchronized cell populations with 0.01% trypsin was found to increase fusion capacity of these interphase cells approximately 2 fold, to a maximum comparable to that seen in the colcemid-arrested mitotic cells. They therefore suggested that variations in fusion capacity may be related to cell cycle-dependent control of the synthesis and/or excretion of glycolipids and glycoproteins which change the properties of cell surface sites. Excretion of glycolipids and glycoproteins was maximal during the late G2 and M portions of the cell cycle (21).

A number of researchers have observed pH- and temperature-dependent aspects of the process of Sendai virus-induced fusion. Early experiments indicated that optimal Sendai virus-induced fusion of Ehrlich ascites tumor (EAT) cells occurred at pH 7.6,

37° C (1,18,22). Croce et al. (23) found that the yield of human x mouse Sendai virus-induced polykaryons and hybrids was several hundred times greater at pH 7.6-8.0 than at the lower pHs (6.8-7.2), the crucial time period for such pH exposure being during the first 4 to 8 days following the original fusion event.

Optimal fusion apparently also requires the presence of certain divalent cations. Okada and Murayama (24) originally demonstrated that Ca^{++} ions were a requirement for syncytia formation among animal cells. Wainberg, Howe, and Godman (25) confirmed this observation using mouse L cells. They also fused human and mouse cells with Sendai virus in the presence of various cation chloride salts and observed that fusion frequency was enhanced when cupric ions were added in concert with the virus particles. More recently, Vollet and Roth (26) found a Ca^{++} ion requirement for fusion of certain marine protozoans.

Hitchcock (27) found that while many cell types show low fusion capacities in suspension, fusion susceptibility is significantly increased when the cells are maintained in monolayers. Klebe et al. (28) also suggested that the suspension method of fusion has certain disadvantages, mainly the difficulty in controlling number of cell contacts. Fusion in suspension increases the likelihood of multicellular fusions and, since such multinuclear cells are largely inviable, reduces the yield of viable hybrids.

The ratio in which the parental cells were mixed was found to have a marked effect on hybridization efficiency in these experiments; hybrid colony frequency was at a maximum when greatly

unequal ratios of parental cells were used and decreased as the ratio approached 1. The use of a large excess of the sensitive or biochemically marked parent in comparison to the insensitive non-selectable parent was desirable in order to increase the ratio of hybrid (selectable) to hybrid (non-selectable) colonies which result after plating (29). Davidson and Ephrussi (30) observed a low mating ratio of normal:drug-resistant parental cells substantially increased the "effective mating rate". Klebe et al. (28) found fusion to be optimal at a ratio of 1:1. Harris (3) however, obtained the best rat lymphocyte x HeLa cell fusion frequencies by using five times as many of the poorly fusing lymphocytes as HeLa cells.

Recently a number of studies have related cell fusion capacity to plasma membrane characteristics (31-33). Poste (31,32) stated that cells with poor fusion capacities tend to have fewer microvilli and significantly thicker glycoprotein cell coats than cells with higher fusion capacities. He suggested that cells with surface coats thicker than 35 Å fuse poorly because they possess too few microvilli to overcome the energy barrier of repulsive electrostatic forces opposing cell-cell contact. Apposition of membranes at distances less than 10 Å is a prerequisite for cell fusion. The opposing energy barrier is then said to be "in the primary minimum," having been overcome by attractive forces (primarily London-van der Waals forces) and by chemical and physical

bonds forming at the newly interacting surfaces (32). Though lymphoid plasma membranes were previously observed to bear numerous microvilli (34), cultured lymphoblasts nonetheless displayed low fusion capacities in our studies.

Rigidity of cell membrane was suggested to be a major characteristic of fusion-resistant cells by a number of researchers. Lymphoid cell membranes were observed to contain high molar ratios of cholesterol:phospholipid (1:1) (35,36). High cholesterol: phospholipid ratios contributed to enhanced membrane rigidity and a fusion-resistant state in primary rhesus monkey kidney (MK) cells which, in contrast to fusion-sensitive baby hamster kidney (BHK) cells, were much less sensitive to osmotic shock, immune cytolysis, and disruption by homogenization (37). The investigators suggested that the more rigid MK membrane was perhaps better able to withstand stresses which occur during fusion or osmotic imbalance (swelling), hence reducing the tendency to participate in membrane-membrane interactions.

Poole et al. (38) hypothesized that lysolecithin enhances fusion by interfering with the integrity of the lipoprotein membrane. They suggested that fusion capacity is enhanced when a membrane organized in the bimolecular leaflet mode (stable) is converted to one of micellar organization (unstable) by agents such a lysolecithin (38,39), certain fatty acids (40), or non-ionic surface-active adjuvants (41).

Originally, the use of virus inactivated with β-propiolactone

or ultraviolet irradiation was thought to be preferable for the formation of somatic cell hybrids to be used in studies of gene regulation, since such inactivation theoretically precluded the possibility of virus-induced chromosomeal, metabolic, or regulatory abnormalities. More recent research has indicated that the so-called "inactive" virus may still be capable of exerting regulatory influences (42,43). The use of lysolecithin is an example of one cell fusion method with which experimenters have attempted to bypass the necessity for using virus. Other non-viral cell fusion methods have included the use of low temperature, digitonin, polylysine, trypsin, and phytohemagglutinin (44), unilamellar lipid vesicles (45), glycerol monooleate (40), and microsurgery (46). These methods have had varying degrees of success and in some cases appear to be uniformly unsuccessful.

Non-viral experiments involving intraspecific fusion of the protozoan Echinosphaerum nucleofilum, a heliozoan characterized by numerous axopodia, indicated that effectors that act to denude existing cell membrane create conditions favorable to fusion (47). Nicolson (48) observed that anionic sites on the surface membrane change topologically after neoplastic transformation, pH changes, or treatment with phospholipase C or trypsin. Since cells that have undergone neoplastic transformation fuse more readily than normal diploid cells (17,31), such cell surface changes may be closely related to cell fusion capacity.

Snow and Allen (49) first demonstrated that trypsin treatment

of baby hamster kidney (BHK) cells caused significant loss of macromolecules containing amino sugars, mucoproteins, proteins, and neutral polysaccharides, and removed approximately 39% of the cell-bound sialic acid. The poor fusion capacity of several lymphoblast cell lines, which can be improved by trypsin treatment (50,51), may thus be related to the presence of cell surface materials which render the membrane refractory to fusion.

Regulatory mechanisms in mammalian cells can be studied by observation of the retention or extinction of a given differentiated function in somatic cell hybrids between differentiated and undifferentiated cells (9,52,53). Differentiated functions in somatic cell hybrids may be completely or partially turned off (54-58), may continue to operate unaffected as in the parent cell (16,59-61), may be enhanced (62,63), or new differentiated functions, possessed by neither of the original parental cell lines, may appear (64-66).

We were particularly interested in the control of immunoglobulin synthesis in established human lymphoblast cultures grown in our laboratory. Long-term cultures of human leucocytes were originally initiated with blood samples from patients with various lympho-proliferative diseases such as Burkitt's lymphoma and lymphocytic leukemia (67); later cells were successfully cultured from normal individuals (68,69). Once established in culture, lymphocytoid cells retained many of their differentiated functions. Lympho-

cytoid cells or lymphoblasts were shown to produce both heavy and light chain immunoglobulins (70-80), some in the form of specific antibody (81), migration inhibitory factor (MIF) (82), interferon, cytotoxic factor, skin reactive factor, and complement (83).

A general mechanism for the control of immunoglobulin biosynthesis in lymphoblast x fibroblast somatic cell hybrids has not been determined, though a considerable amount of data is available. Periman (84) fused an IgA- and lambda chain-producing mouse plasmacytoma cell line with a thymidine kinase (TK) deficient mouse L cell line. The resulting hybrids produced both IgA and lambda chains, but in smaller amounts than the plasmacytoma parent. This reduction was thought to be due to an inefficient translation or secretion mechanism in the hybrid. Orkin et al. (85) found that hybrids of lambda chain-producing human lymphoblasts and mouse fibroblasts continued to produce human lambda chains, but did not synthesize human heavy chains or mouse immunoglobulins. Coffino et al. (86), in contrast, found that immunoglobulin synthesis was suppressed in mouse myeloma x mouse fibroblast hybrids. Mohit and Fan (87) observed that hybrids of IgG- and kappa chain-producing mouse myeloma cells and non-immunoglobulin-producing mouse lymphoma cells continued to synthesize kappa chains, but ceased to produce IgG heavy chains. Cotton and Milstein (88) hybridized rat

myeloma x mouse myeloma cells and observed continued production of both rat and mouse immunoglobulin, as well as immunoglobulin molecules containing one light chain of each parental type. Schwaber and Cohen (89,90) found that mouse myeloma x non-immunoglobulin-producing human lymphocyte hybrids produced both mouse and human immunoglobulins, suggesting that the differentiated murine genome turned on the previously inactive human genes. The existing data on control of immunoglobulin synthesis thus leaves the problem unresolved.

Our study defined viral and non-viral factors affecting fusion of human lymphoblasts, some of which were known immunoglobulin producers. The differentiated capabilities of hybrids formed from parental cells displaying varied differentiated functions were characterized in preliminary studies.

MATERIALS AND METHODS

Lymphoblast Cloning Procedure

The procedure used to clone human lymphoblasts in our laboratory was similar to that used by Coffino, Laskov, and Scharff (91) and was basically a modification of Puck's technique for the cloning of HeLa cells (92). The method utilized a basal layer of fibroblastic "feeder" cells, a separating layer of agarose-R.P.M.I. 1640 medium, and an uppermost agarose-medium layer containing the experimental cells.

Soft Agar Plating Using Feeder Layers

1. Fibroblastic feeder layer cells were allowed to grow to near confluence in Eagle's minimum essential medium (MEM) + 11% fetal calf serum (FCS) in 60 mm Petri dishes.

2. R.P.M.I. 1640 medium supplemented with 20% FCS was freshly prepared and warmed to 38^{o} C.

3. A 2.4% stock solution of agarose was prepared using 0.48 g agarose in 20 mls 0.85% saline. The solution was autoclaved for 20 minutes at 15 pounds of pressure and used hot in Step 5 below.

4. The cell culture to be cloned was counted and calculations made for the desired final cell concentrations. Final cell concentrations usually ranged from 50-5000 cells per feeder layer plate.

5. The 2.4% agarose stock solution was diluted 1:11 and 1:10 in the warmed R.P.M.I. 1640 + 20% FCS to yield respectively 0.22% and 0.24% agarose-medium. Agarose-medium was then cooled to 38° C.

6. Medium was aspirated off the feeder layer Petri dishes with a Pasteur pipet. The feeder layers were then overlaid with 2.5 mls of 0.24% agarose-medium per Petri dish. The agarose-medium overlays were cooled briefly at room temperature and placed in a 37° C CO_2-supplemented incubator during the preparation of the experimental cell aliquots.

7. The cells to be cloned were then diluted in R.P.M.I. 1640 medium such that the desired final number of cells per Petri dish was contained in 0.1 ml aliquots. 0.1 ml of a given cell dilution was pipetted into a capped plastic test tube for each Petri dish (0.2 ml cell suspension for 2 Petri dishes, 0.3 ml cell suspension for 3 Petri dishes, etc.).

8. 2.0 mls of 0.22% agarose-medium were added to these tubes for each 0.1 ml cell suspension. The cells were then mixed by gentle pipetting.

9. 2.5 mls of the 0.22% agarose-medium cell suspension were layered dropwise over the 0.24% agarose-medium layer in each of the feeder layer Petri dishes. The final agarose-medium layer was cooled briefly (5-10 min) at room temperature and the Petri dishes were stored in a 37° C, CO_2-supplemented incubator.

10. Cells were fed 2-3 hours following plating with 1.0 ml R.P.M.I. 1640 + 20% FCS per Petri dish and at 5-6 day intervals thereafter with 0.5 ml R.P.M.I. 1640 + 20% FCS.

Isolation of Colonies

1. Petri dishes were examined within a few hours of plating and positions of lymphoblast cells observed to be lying close together or in close proximity were marked with wax pencil on the bottom of the dish. Colonies developing from such marked cells were assumed to be the progeny of more than one cell and were not picked.

2. Putative clonal colonies of lymphoblasts appeared following 3-4 weeks of incubation and re-feeding. Desired colonies were isolated with a cotton-plugged Pasteur pipet and a small rubber bulb. All manipulations were easily performed under a dissecting microscope at 7X magnification in a tissue culture transfer hood.

3. Isolated colonies were initially transferred to 30 mm Petri dishes containing 1.0 ml R.P.M.I. 1640 medium supplemented with 14.3% FCS. Colonies which grew were later transferred to T-30 Falcon tissue culture flasks.

Hybrid Colony Selection and Maintenance

After a growth period of 3-4 weeks in selective medium, hybrid colonies were picked from the 60 mm Petri dishes by scraping. The scraping technique employed a sharp, bevel-edged small teflon scraper attached to a pair of forceps which could be sterilized by immersion in 95% ethanol and flaming. The scraper was used to

gently detach the fibroblastic colony from the plate surface. The colony was then sucked up with a cotton-plugged Pasteur pipet and transferred to a 30 mm Petri dish containing 1.0 ml MEM + 11% FCS supplemented with 0.05 ml 2 x 10^{-3}M hypoxanthine and 0.01 ml 4 x 10^{-3}M thymidine. Cultures of hybrid cells were transferred to larger containers when they reached near-confluency. They were fed at weekly intervals in the first month with hypoxanthine- and thymidine-supplemented medium (30).

Cell Freezing Procedure

New lymphoblast and hybrid clones were frozen as soon as they reached a sufficiently high population level since human and mouse hybrids often lose chromosomes at a rapid rate (93). Cells were frozen at densities of 2-5 x 10^{6} cells/ml following centrifugation for 10 min at 2000 rpm at 4^{o} C. Cell pellets were resuspended in glycerol freezing medium (R.P.M.I. 1640 + 10% sterile glycerol + 18% FCS) and the cell suspensions were injected into ampoules using sterile syringes. Ampoules were then flame-sealed and stored at -93^{o} C in the Revco.

Chromosome Procedures

Fibroblast Chromosome Spreads

Chromosome spreads from fibroblastic cells were prepared from cells growing attached to coverslips in 30 mm Petri dishes. Colchicine (0.5 ug/ml) was added to each Petri dish to block cells in the metaphase of mitosis and the cells were incubated

for approximately three hours. Cells were rinsed once at the end of the three hour colchicine block with cold hypotonic 0.075M KCl, allowed to incubate in 1.0 ml hypotonic KCl for 30 min at 4° C, and then fixed in three changes of 3:1 methanol:acetic acid fixative for successive periods of 5 minutes, 10 minutes, and 15 minutes. The cells were then fixed in 5-10 drops of 1:2 methanol:acetic acid for ten minutes since this more volatile mixture facilitated chromosome spreading. The coverslips were then removed from the Petri dishes, dried briefly on a hot plate (100-125° C) to further promote chromosome spreading and the cells were stained in 1:4 Leishman stain for 3 minutes.

Lymphoblast Chromosome Spreads

Lymphoblast chromosome spreads were prepared by methods similar to those described above with logarithmically growing lymphoblast cells. Since these cells grew in suspension, the fdlowing procedural modifications were necessary. Colchicine stock solution was added to the lymphoblast suspensions at a final concentration of 0.1 ug/ml and the cultures were incubated for approximately 2-4 hours. The cells were then centrifuged for 6 minutes at 1500 rpm, resuspended in 10 mls of room temperature hypotonic 0.075M KCl and incubated for 20 minutes. They were then recentrifuged for another 6 minutes at 1500 rpm and all but 1.0 ml of the hypotonic KCl was removed by suction. An equal volume of cold 3:1 methanol:acetic acid was added to the cells

and the suspension was centrifuged for 5 minutes at 4° C and 1500 rpm. The fixative was then removed, 2.0 mls of cold 3:1 methanol:acetic acid added, and the centrifugation step repeated. The final pellet was suspended in 10 drops of cold 2:1 methanol: acetic acid. Chromosome spreads were obtained by placing one drop quantities of fixed cells on glass slides with a Pasteur pipet, allowing the suspension to spread, and then gently flaming the slides until dry. Cells were stained in 1:4 Leishman stain for 2½ to 3 minutes.

Immunofluorescence Techniques

Monospecific fluorescein isothiocyanate-conjugated sheep and goat anti-human IgG, IgM, IgA, lambda chain, and kappa chain were obtained from Meloy Laboratories. Non-conjugated monospecific sheep anti-human IgG, IgM, IgA, lambda chain, and kappa chain were obtained from the same source, as was fluorescein-conjugated rabbit anti-sheep IgG. Only cells in the logarithmic phase of growth were used in these studies since actively growing cells are known to display maximum immunoglobulin synthesis (94-96). Cells were prepared by two different methods for fluorescent antibody staining.

1. Cytoplasmic Fluorescence

For cytoplasmic fluorescent staining, samples of approximately 10^7 cells were centrifuged at 1500 rpm for 5 minutes and the resulting pellet rinsed twice in cold 0.85% saline. The pellet was resuspended in cold 1:1 acetone:alcohol, fixed for 30 minutes,

and recentrifuged. The cells were then resuspended in 10 drops 1:1 acetone:alcohol and spotted on coverslips with a Pasteur pipet. Dry coverslips could be stored for an indefinite period until ready for use.

Prior to immunofluorescent staining the cells on the coverslips were rehydrated for 30 minutes in cold 0.85% saline in Coplin jars. One drop of anti-human immunoglobulin, diluted 2- to 10-fold in 0.85% saline, was spread over the surface of the coverslip and the cells were placed in a 37^{o} C water-jacketed incubator (supplemented with 5% CO_2) for one hour. The coverslips were then incubated in three rinses of phosphate-buffered saline (PBS) for ten minutes per rinse and mounted in 1:3 PBS:glycerol on glass slides for observation.

2. Surface Fluorescence

The surface fluorescence technique of Pernis et al. (97) eliminated the problem of cell clumping often seen on coverslips prepared by the cytoplasmic fluorescence method, and gave generally better results than method 1. Aliquots of 15-20 x 10^6 cells were centrifuged at room temperature for 10 minutes at 1000 rpm. The pellet was resuspended in 2.0 mls of 5% BSA in PBS and equal volumes of the cell suspension and precooled diluted antisera were mixed in a test tube and placed in an ice bath for 30 minutes. The cells were then rinsed three times with cold 5% BSA in PBS, resuspended in one drop of 1:3 PBS:glycerol and mounted on glass slides.

An indirect immunofluorescent method was also used for some of our studies in which the cells were first treated with non-fluorescein-conjugated sheep anti-human serum, then rinsed three times with 5% BSA in PBS to remove unbound antibody, and finally treated with fluorescein-conjugated rabbit anti-sheep IgG. This method was not successful due to high background fluorescence.

Controls used for determining antibody specificity in our immunofluorescent assays included: non-immunoglobulin-producing human fibroblast cells, immunoglobulin-producing mouse lymphoblast cells, and human lymphoblast cells that had been "blocked" with undiluted non-conjugated anti-human serum prior to treatment with the fluorescein-conjugated antiserum.

Cells were observed at 500X magnification on a Zeiss microscope using a darkfield condenser and a 100 watt quartz-iodide or mercury ultraviolet light source.

Preparation of Cell Extracts for Immunological Analyses

Cell extracts were prepared from approximately 10^7 cells harvested in logarithmic growth and centrifuged for 5 min at 1500 rpm. The pellet was rinsed twice in 0.025u ionic strength barbital buffer (the 0.075M barbital stock solution had an ionic strength of 0.10u, pH 8.2, and a conductance in the range of 4-5 x 10^3 umhos) (see Table 1), transferred to 1.0 ml plastic centrifuge tubes and finally resuspended in 50 ul of 0.025u barbital buffer containing 2% Triton X. This suspension was sonicated three times in five second bursts using the microtip attachment of a Bronwill Biosonik

IV sonicator set on low and the #20 setting. The sonicated suspension was then centrifuged in the cold at 7000 x g for 30 minutes and the resultant clear extract removed from the debris with a drawn-out Pasteur pipet.

In an alternative method used to remove cell lipids, the cells were transferred to 2.0 ml glass tubes after the first 0.025u barbital buffer rinse, resuspended in 100 ul of 0.025u barbital buffer and sonicated for three five second bursts. An equal volume (100 ul) of Genesolv-D (trichloro-trifluoro-ethane) was added, the tube was agitated for 30 seconds and then centrifuged at 20,000 rpm in a Sorvall centrifuge for 20 minutes at 4° C. Two distinct layers were formed following centrifugation, the top layer containing the cell extract and the bottom layer retaining cell lipids. The top layer was removed with a drawn-out Pasteur pipet.

Electroimmunodiffusion

The electroimmunodiffusion (E.I.D.) technique used in our laboratory to assay quantitative production of immunoglobulin is a modification of that described by Lopez, Tsu, and Hyslop (98). Sheep anti-human IgM and IgA used in the E.I.D. procedure were dialyzed prior to use against 4-6 liters of 0.025u barbital buffer + 0.1% sodium azide. Human immunoglobulin reference standards were obtained from Meloy Laboratories and contained 17.1 mg/ml IgG, 2.91 mg/ml IgA, and 1.53 mg/ml IgM. The antigen standards were diluted in 0.025u barbital buffer to give

concentrations of 20,40,60,80, and 100 ug/ml for IgA and IgM (IgG production was not investigated using the E.I.D. technique).

E.I.D. Procedure

1. Pre-coated duffusion plates were prepared by lightly coating clean glass plates with 0.2% agarose and refrigerated until needed.

2. 1.0% agarose in 0.025u barbital buffer was prepared, cooled to approximately 40° C in a water bath, and 6.0 ml aliquots pipetted into glass test tubes. Dialyzed sheep anti-human immunoglobulin of the desired type was then added to the 1.0% agarose in aliquots ranging from 60-100 ul, an antiserum dilution range of 1-2%. The precoated glass diffusion plates were overlaid with the antiserum-containing 1.0% agarose aliquots and allowed to cool at room temperature.

3. Agarose wicks and bridges were poured from 1.5% agarose prepared in 0.025u barbital buffer. Such wicks and bridges were found to be necessary in the IgM experiments to retard flow of water across the diffusion plate. They were also used in the IgA experiments.

4. Wells were cut ½ inch from the bottom of the antiserum-containing 1.0% agarose overlay sheets on the glass diffusion plate. These were filled with 5 ul aliquots of chilled reference standard dilutions and cell sample extracts.

5. Buffer troughs were filled with cold 0.10u barbital buffer. The E.I.D. apparatus was attached to a cold (0-4° C)

water-circulating condensor and to a power source in such manner that antigen migration proceeded from - to + at a voltage of 300 and a current of 45-55 milliamps.

6. Both IgM and IgA experiments were run for 6 hours. At the completion of the run, diffusion plates were removed from the E.I.D. apparatus and dried overnight.

Staining Procedure

1. Dried diffusion plates were fixed for 10 minutes in 5:5:1 methanol:water:glacial acetic acid.

2. Fixed plates were rinsed for 1-2 hours in distilled water.

3. After rinsing, plates were stained for 10-15 minutes in 1.0% Coomassie Blue + 5% acetic acid.

4. Finally plates were rinsed several times in the fixing and destaining solution (5:5:1 methanol:water:glacial acetic acid). Peak heights were measured and recorded.

TABLE 1

I. Barbital Buffer (0.075M Veronal Buffer)

15.85 g sodium barbital

770 mls glass distilled water

230 mls 0.1N HCl

Final pH was 8.2 without adjustment.

II. Fixing and De-Staining Solution (5:5:1)

1000 mls methanol

1000 mls glass distilled water

200 mls glacial acetic acid

III. Coomassie Blue Stain

2 g Coomassie blue

190 mls glass distilled water

10 mls glacial acetic acid

The stain (1% stain in 5% glacial acetic acid) was filtered prior to use.

Table 1. Composition of solutions used in the electroimmuno-diffusion procedure.

Figure 2. Electroimmunodiffusion of IgM reference standards. This standard curve is the result of three 1.75% anti-IgM diffusion plate concentration E.I.D. runs using new Meloy antigen reference standards.

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FIGURE 2

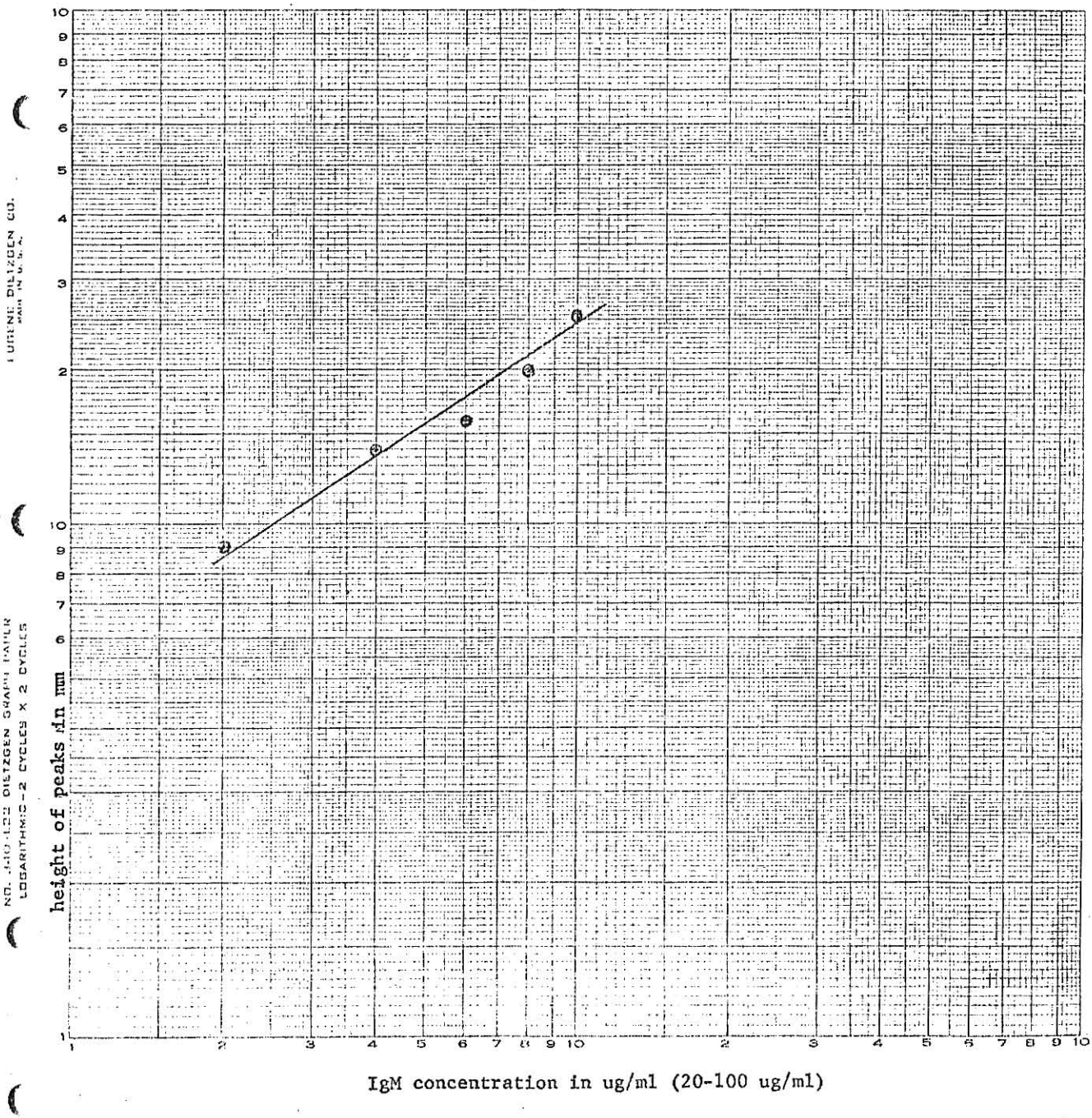
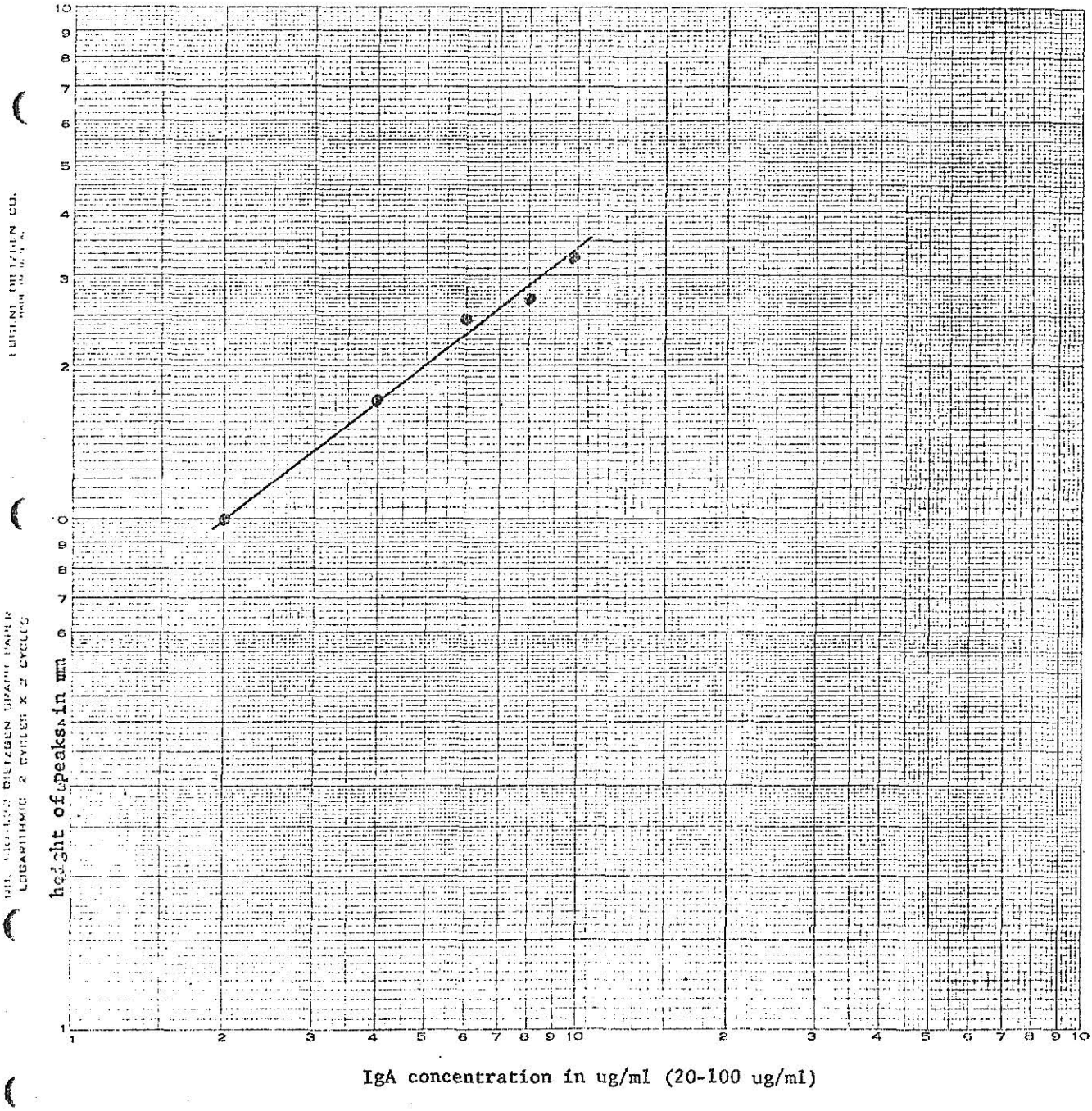


Figure 3. Electroimmunodiffusion of IgA reference standards. This standard curve is the result of three 2.0% anti-IgA diffusion plate concentration E.I.D. runs using new Meloy antigen reference standards.

FIGURE 3



Plating Efficiencies of Cell Lines and Selection of Clones

Attempts were made to clone MGL8 and the other diploid lymphoblast lines by the soft agarose feeder layer method (91,92), by a microwell dilution technique (100) and by attempted isolation of single lymphoblast cells in sterile 5 ul capillary tubes (101). Only the first of these three methods resulted in colony growth after dilution of the cell population.

Diploid human lymphoblasts were difficult to clone in comparison to diploid human fibroblasts or to aneuploid human lymphoblast lines. Of the tested lymphoblast lines, MGL5 had the lowest plating efficiency of 5×10^{-4} (an average of 5 colonies per 10,000 cells plated). MGL7 had a plating efficiency range of 4×10^{-4} to 5×10^{-3} (0.04-0.5%), while MGL8, the most easily cloned of the diploid human lymphoblast lines, was found to have a plating efficiency of 3.8-5.0%. Successful colony growth from cells plated in agar was not observed with the MGL6 line. In contrast, the aneuploid MGL33C19 lymphoblast line had a plating efficiency which varied from 26.3% to 50% in more recent experiments, while the KS2 human fibroblast line had a plating efficiency of approximately 14%.

Colonies isolated from MGL5 and MGL7 cloning experiments repeatedly did not grow after transfer to liquid medium in 30 mm Petri dishes and died after 3-4 weeks. Colonies of the MGL8 line, however, survived in a high percentage of transfers. The

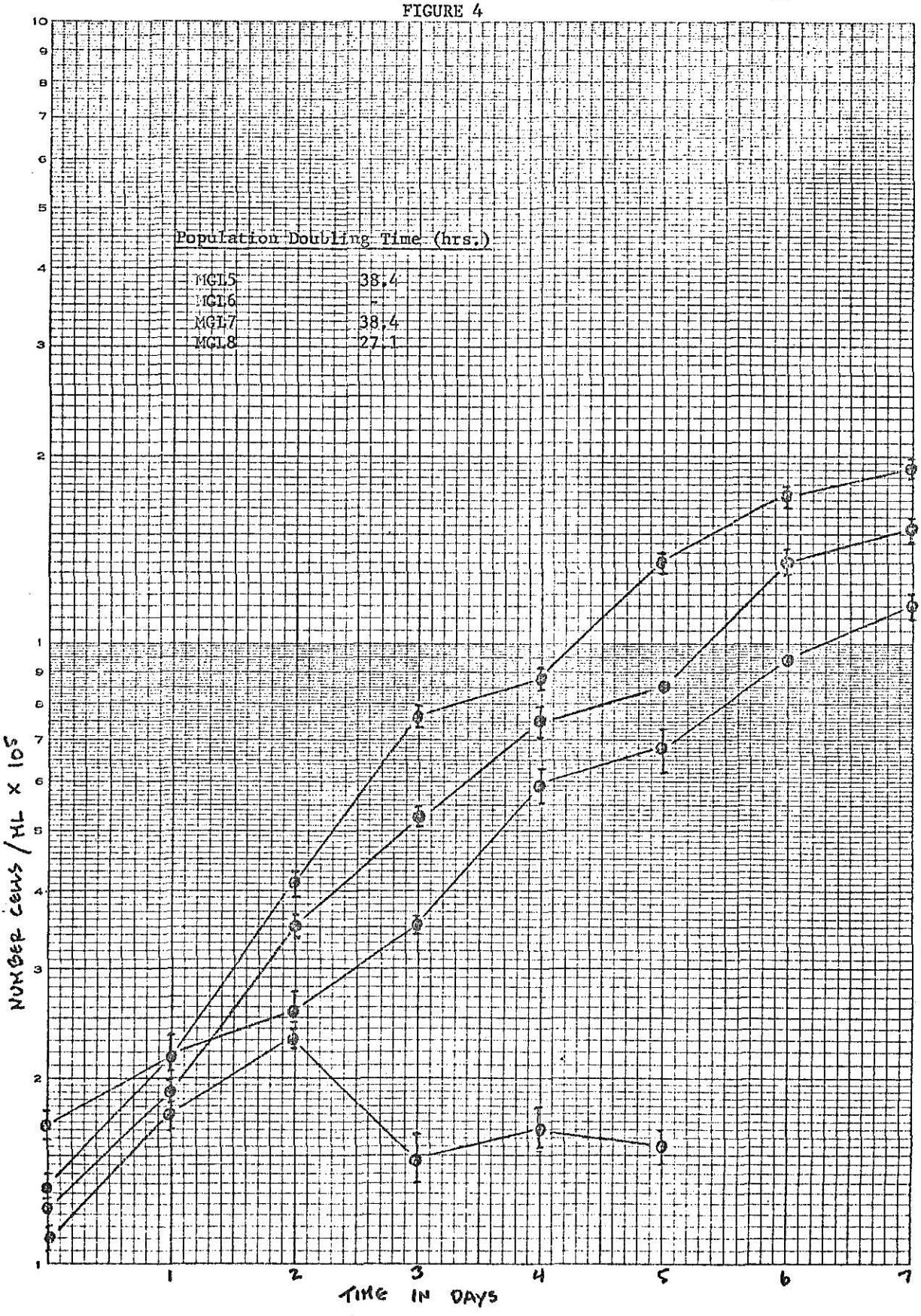
RESULTS

Characterization of Lymphoblast Cell Cultures

Initial experiments were aimed at determining the growth rates and saturation densities of four lymphoblast cell lines obtained from Dr. J.W. Littlefield at the Massachusetts General Hospital. These cell lines were selected because they possessed diploid chromosome numbers and had been grown for only a brief time in tissue culture obviating the chromosomal instability problems inherent in older (1-2 year) lymphoblast cultures (99). The growth curves for these cell lines (Figure 4) indicated that the MGL8 cell line had the most rapid growth rate (a doubling time of 27.1 hours) and attained the highest final cell concentration. The MGL6 cell line, exhibiting the least rapid growth rate, was originated from a male patient with a 4/9 chromosomal translocation and contained a small ring chromosome. It has grown poorly since it was obtained by our laboratory, seldom reaching a density of more than 3-4 x 10^5 cells/ml. It was not used in any of the fusion or hybridization experiments. On the basis of this growth rate data, MGL8 was chosen as the cell line most likely to produce colonies after plating in the agarose-solidified media required for the isolation of clonal cell populations with desired genetic uniformity for further studies.

Figure 4. Growth curves of four human lymphoblast cell lines in R.P.M.I. 1640 medium supplemented with 14.3% fetal calf serum (heat-inactivated).

FIGURE 4


Population Doubling Time (hrs.)
MGL5 38.4
MGL6 -
MGL7 38.4
MGL8 27.1
NUMBER CELLS / ML X 10⁵
TIME IN DAYS
EUGENE DIETZGEN CO.
MADE IN U.S.A.
NO. 340-L210 DIETZGEN GRAPH PAPER
SEMI-LOGARITHMIC
2 CYCLES X 10 DIVISIONS PER INCH

largest and most rapidly growing MGL8 colony, designated MGK8E, was obtained from a plate initially seeded with 200 cells which yielded 3 colonies after 4 weeks of incubation. The MGK8E colony was widely separated from the other colonies on the plate and grew up in a vicinity in which no cells were originally observed to be lying close together. It was therefore labelled a clonal cell population, the progeny of a single MGL8 cell.

Effect of Fusion Conditions on Cell Survival and Hybrid Frequency

The selective system used in our hybridization experiments was a half-selective system, based on rinsing away all non-fused normal human lymphoblast parental cells, which, unlike the enzyme-deficient fibroblast parental cells, would otherwise grow in HAT medium. A series of experiments was initiated to determine whether any of the treatments during the fusion process might be specifically killing fibroblastic cells and hybrids of fibroblastic morphology prior to plating and HAT selection. Possibly the enzyme-deficient mouse and hamster cell lines we used were particularly sensitive to either abrupt temperature changes or to prolonged periods of incubation in the presence of Sendai virus. 1×10^6 3T3c2 mouse cells suspended in 1.0 ml of Hanks basal salt solution (HBSS) either with or without 2500 HAU Sendai virus were carried through the fusion process and four 60 mm Petris of 200 cells per plate in MEM + 11% FCS were plated at 10 minute intervals. The initial pre-HBSS plates were not suspended in HBSS, but simply harvested, counted, diluted in medium, and re-plated. At one week, plates

were fixed in formalin:PBS and stained in 1:50 Giemsa. The results, summarized in Table 2, showed that there was approximately a 16% reduction in fibroblast viability following the hybridization treatment. This slight decrease in viability was insufficient, however, to explain the complete failure of all the lymphoblast x fibroblast experiments to produce hybrid colonies.

Further experiments were set up to investigate the effect of medium type and fetal calf serum concentration on fibroblast survival. Results (Tables 3 and 4) indicated that the plating efficiency and growth ability of mouse and hamster fibroblasts were not greatly altered by varying media and serum concentrations.

Characterization of Somatic Cell Hybrids

Selection and Identification of Human Lymphoblast x Mouse Fibroblast Hybrids

Viable hybrids were identified by: 1) the ability to grow and form fibroblastic colonies in HAT medium, 2) a fibroblastic morphology which differed from the "epithelial" cell appearance of the 3T3c2 parental cells, and 3) increased chromosome numbers, and 4) the presence of human chromosomes in the hybrid chromosome spreads. We observed early in these studies that hybrids would disintegrate if not maintained in 1×10^{-4}M hypoxanthine and 4×10^{-5}M thymidine for the first few weeks after transferring colonies as previously reported by Davidson and Ephrussi (30). Newly isolated hybrids growing in HAT medium are thought to

TABLE 2

Cell Treatment	#Cells/Plate	Average # Colonies	Average Plating Efficiency
pre-HBSS suspension	400	137	34.3%
post-HBSS suspension	200	53	26.5%
0' - Sendai	200	40.5	20.3%
10', 4° C - Sendai	200	37	18.5%
20', 4° C - Sendai	200	48.5	24.3%
10', 37° C - Sendai	200	48.5	24.3%
20', 37° C - Sendai	200	33.8	16.9%
0' + Sendai	200	53.5	26.8%
10', 4° C + Sendai	200	39.5	19.6%
20', 4° C + Sendai	200	42.5	21.3%
10', 37° C + Sendai	200	42	21.0%
20', 37° C + Sendai	200	38	19.0%

Table 2. Effect of Sendai Virus and Temperature Conditions on the viability of 3T3c2 mouse fibroblast cells as determined by plating efficiencies.

TABLE 3

Medium	Total # Cells Plated	Average # Colonies	Average Plating Efficiency
MEM + 9% FCS	800	42.3	21.1%
MEM + 15% FCS	800	45.3	22.5%
MEM + 20% FCS	800	37.5	18.7%
R.P.M.I. 1640 + 15% FCS	800	53.8	26.8%

Table 3. Plating efficiency of 3T3c2 mouse fibroblasts in various media and serum concentrations. 200 cells were plated per 60 mm Petri.

TABLE 4

Medium	Total # Cells Plated	Average # Colonies	Average Plating Efficiency
Dulbecco's Medium + 11% FCS	2760	136.4	39.8%
MEM + 11% FCS	2760	133.4	39.5%
F12 Medium + 11% FCS	2760	108.4	31.5%

Table 4. Plating efficiency of TG2 hamster fibroblasts in various media. Cell densities per 60 mm Petris were 230 cells per plate in Experiment 1 and 460 cells per plate in Experiment 2.

develop a transient dependence on exogenous hypoxanthine and thymidine which presumably disappears with the loss of aminopterin from the cell and the resynthesis of folate reductase (44).

The morphology of mouse 3T3c2 cells differed markedly from that of the lymphoblast x 3T3c2 hybrids isolated. The hybrids obtained had an elongated "spindle-shaped" morphology more characteristic of human fibroblastic cells than the compact, triangular, epithelial-like morphology of 3T3 cells. The change in morphology is especially interesting since lymphoblasts normally grow in suspension, whereas the hybrids obtained grew attached to the Petri dish surface as did the parental mouse cells.

Chromosome numbers of hybrid cells varied according to the specific hybrid clone examined, although we generally observed relatively higher modal chromosome numbers in the hybrids than in the parental cells. The MGK8E lymphoblast parent had a normal diploid number of 46 chromosomes and the 3T3c2 mouse cells had a modal number of 56 chromosomes in at least 20 cells of each culture examined. Hybrid (MGK8E x 3T3c2) H8EB chromosome numbers varied from 61 to 109. The HLPM-1 hybrid, resulting from fusion of human LPM 10-20 lymphoblast cells (46 chromosomes) and 3T3c2 mouse cells, had 86 to 148 chromosomes. Metacentric human chromosomes could be easily identified in hybrid cells (Figure 5) since the 3T3c2 parent had all acrocentric chromosomes characteristic of mouse cells. Karyotype analyses

Figure 5. Chromosome spread of a human x mouse hybrid cell in which human chromosomes can be identified.

FIGURE 5

were not performed on all hybrid cells obtained since our initial goal was to begin to characterize immunoglobulin production by hybrids as soon as possible after isolation before extensive loss of human chromosomes occurred. Our current chromosome analyses of hybrids are thus preliminary estimates since human acrocentric chromosomes are morphologically indistinguishable from mouse acrocentric chromosomes and require additional techniques, such as chromosome banding procedures, for identification. Table 5 lists the parental cell lines and experimental treatments used to induce hybrids obtained in these studies. These hybrids are currently stored in a Revco freezer pending further investigation.

Detection of Immunoglobulin Production by Lymphoblasts and Hybrids with Immunofluorescence

Characterization of immunoglobulin production by lymphoblasts involved determination of either cytoplasmic fluorescence or surface fluorescence following procedures described in the Materials and Methods section for treatment of cells with fluorescein-conjugated antisera against human immunoglobulins. Because cell clumps and cell debris were found to display bright, nonspecific fluorescence, only single cells were characterized in our studies.

Lymphoblast lines studied for immunoglobulin production by observation of cytoplasmic fluorescence in ethanol-acetone fixed cells reacted to fluorescein-conjugated monospecific antisera as shown in Table 6. The validity of these results was questioned when end-point dilution experiments utilizing monospecific antisera

TABLE 5

Hybrid Clone	Source	Fusion Method	# Colonies on Original Plate
H8EB	MGK8E x 3T3c2	Sendai	1
H8ED	MGK8E x 3T3c2	Sendai	3
H8EE	MGK8E x 3T3c2	trypsin	14
H8EF	MGK8E x 3T3c2	trypsin	12
H8EG	MGK8E x 3T3c2	trypsin	12
HMG57-1	MGL57-22 x 3T3c2	Sendai	2
HMG57-2	MGL57-22 x 3T3c2	trypsin	2
HMG57-3	MGL57-22 x 3T3c2	spontaneous	2
HMG57-4	MGL57-22 x 3T3c2	spontaneous	7
HMG57-5	MGL57-22 x 3T3c2	spontaneous	7
HMG57-6	MGL57-22 x 3T3c2	spontaneous	7
HLPM-1	LPM10-20 x 3T3c2	Sendai	2
HLPM-2	LPM10-20 x 3T3c2	spontaneous	2
HLPM-3	LPM10-20 x 3T3c2	spontaneous	2
HLPM-4	LPM10-20 x 3T3c2	Sendai	3
HLPM-5	LPM10-20 x 3T3c2	Sendai	3
HLPM-6	LPM10-20 x 3T3c2	Sendai	3

Table 5. Sources of available human x mouse hybrid clones.

TABLE 6

Cell Line	Reaction to Fluorescein-Conjugated Antisera Specific To:			
	IgG	IgM	IgA	Kappa Chain
MGL5	-	-	-	±
MGL6	+	-	+	+
MGL7	+	-	-	-
MGL8	+	+	+	+
MGK8A	+	+	±	+
MGK8E	+	+	+	+
T-5-1	+	+	+	±
MGL33C19	+	+	±	+
KS4	-	+	-	+
LPM 10-4	±	+	N.T.[1]	-
MGL57	+	-	+	+
B189	+	+	±	+

Table 6. Immunoglobulin production by human lymphoblast cell lines and control cells as determined by cytoplasmic immunofluorescent staining with monospecific fluorescein isothiocyanate-conjugated anti-human heavy or light chain antisera.

[1] Not tested.

diluted as much as 1:400 in 0.85% saline still resulted in distinct fluorescence. Additionally, known non-immunoglobulin-producing lymphoblast control cells and cells blocked with non-fluorescein-conjugated antiserum continued to fluoresce. Fibroblastic control cells were negative or had very weak background fluorescence as was expected for non-immunocompetent cells. Adsorption of the fluorescein-conjugated antiserum against known non-immunoglobulin-producing human lymphoblast cells was attempted to eliminate the non-specific binding, but this was not successful and known negative lines continued to give false positive results. Indirect surface immunofluorescence was attempted by treating the cells with non-conjugated monospecific sheep anti-human immunoglobulin followed by fluorescein-conjugated rabbit anti-sheep immunoglobulin but this was also unsuccessful.

The difficulties with cytoplasmic fluorescence prompted us to use the surface fluorescence staining technique described by Pernis et al. (97). This technique proved superior and resulted in reduced background fluorescence, little or no cell clumping, and considerably less debris and particulate matter associated with the cells. Cellular fluorescence was seen with the characteristic speckled, ring, and cap fluorescence patterns that have been cited as typical of immunoglobulin-producing cells (96,102-104). Control cells which were blocked with non-conjugated antiserum and known non-immunoglobulin-producing lymphoblast and fibroblast

cells showed only a weak background fluorescence. Some revised results from lymphoblast lines previously tested with the cytoplasmic fluorescence technique can be seen in Table 7. MGL8 and MGK8E, for example, appeared to be uniformly negative, as did the five tested MGK8E x 3T3c2 human x mouse hybrids (H8EB, H8ED, H8EF, and H8EG). MGL7 appeared to be strongly positive for IgM, positive for kappa light chain production and weakly positive for lambda light chain production. The T-5-1 lymphoblast line was positive for IgG and lambda chains; KS3 was positive for IgG, IgM, and lambda chains. Contradictory results obtained from surface and cytoplasmic fluorescence methods were probably due in part to the subjective aspect of the immunofluorescent technique (105). Attempts were made therefore to characterize human lymphoblast and hybrid immunoglobulin production by more objective and quantitative methods.

Detection of Immunoglobulin Production by Lymphoblasts and Hybrids with Quantitative Diffusion Methods

Ouchterlony diffusion plates (106) were initially tested as an objective and standardizable method for characterizing human lymphoblast immunoglobulin production. Repeated experiments clearly demonstrated the monospecificity of the experimental antisera but precipitates with cell extracts were not observed. These negative results suggested that the cells in question either did not produce immunoglobulin or produced immunoglobulin in

TABLE 7

Cell Line	Reaction to Fluorescein-Conjugated Antisera Specific To:				
	IgG	IgA	IgM	Lambda Chain	Kappa Chain
MGL5	-	-	-	-	-
MGL7	-	-	+	±	+
MGL8	-	-	-	-	-
MGL8E	-	-	-	-	-
MGL57-22	-	+	N.T.[1]	N.T.	N.T.
LPM 10-20	-	-	+	-	-
T-5-1	+	-	-	+	-
KS3	+	-	+	+	-
H8EB	-	-	-	-	-
H8ED	-	-	-	-	-
H8EF	-	-	-	-	-
H8EG	-	-	-	-	-

Table 7. Immunoglobulin production by human lymphoblast cell lines and control cells as determined by surface immunofluorescent staining with monospecific fluorescein isothiocyanate-conjugated anti-human heavy or light chain antisera.

[1]Not tested.

quantities too low to be detected by the Ouchterlony technique. A more sensitive electroimmunoassay termed electroimmunodiffusion (E.I.D.) (98) was next employed for detection of immunoglobulin production and standard curves for known IgM and IgA concentrations prepared (see Materials and Methods). Experimental runs of cell extracts from the MGL8 and MGK8E lymphoblast lines indicated that these cells did not make IgM since no antigen-antibody precipitate peaks were observed. Extracts of the MGK8E x 3T3c2 human x mouse hybrid lines, H8EB, H8ED, H8EF, and H8EG, were also non-IgM-producers as expected. Several additional experiments with other cell lines indicated that the LPM 10-20 lymphoblast line produced IgM, although an LPM 10-20 x 3T3c2 hybrid (HLPM-1) did not. IgA studies with known IgA-producing lymphoblast cells (MGL57-22) and with MGL57-22 x 3T3c2 hybrids were inconclusive due to problems with nonspecific "tracking."

The major problem encountered with the E.I.D. technique was a nonspecific tracking of materials present in the extract which tended to obscure specific antigen-antibody peaks. This was further complicated by the presence of darkly staining cell debris which accumulated around the sample wells and interfered with antigen migration as evidenced by irregular peaks. The problems were overcome by the use of Genesolv-D (trichloro-trifluoro-ethane) in the extraction procedure and by centrifuging the final extract at 20,000 rpm to remove cell lipids. The solution of these

extraction and immunoglobulin characterization problems should thus contribute to further studies with the hybrids produced in these experiments.

The initial goals of our study to characterize the differentiated capabilities of human lymphoblast x mouse fibroblast hybrid cells were only partially realized due to the problems encountered with cell fusion, hybridization, and immunological analyses discussed previously. Thus, although a number of MGK8E x 3T3c2 hybrids were obtained due to initial indications that MGK8E synthesized IgG, IgM, and IgA (Table 6), further studies with more refined immunological methods indicated that the parental MGK8E cell line was incapable of detectable Ig synthesis (Table 7). It is not surprising, therefore, that the hybrids of MGK8E and non-immunoglobulin-producing mouse fibroblasts likewise do not produce immunoglobulin.

Preliminary electroimmunodiffusion analyses indicated that the MGL57-22 and the LPM 10-20 lymphoblast lines produced IgA and IgM respectively. A hybrid clone of LPM 10-20 x 3T3c2 (HLPM-1) produced in this study and tested by the E.I.D. technique was apparently negative for IgM production. This result may indicate repression of differentiated human gene activity by a bacterial-type repressor molecule from the undifferentiated mouse cell, although more work needs to be done to confirm this negative result. Additional human x mouse hybrid clones of both MGL57-22

and LPM 10-20 were produced near the end of this study and are now available for immunological analyses which may lead to further elucidation of the mechanism controlling immunoglobulin synthesis in human x mouse hybrids. These studies remain important due to the continuing controversy over Ig control mechanisms in human cells.

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FUSION AND HYBRIDIZATION OF HUMAN LYMPHOBLAST CELLS

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The goals of this study were twofold: first, to develop effective methods for induction and isolation of human lymphoblast somatic cell hybrids; and second, to characterize the differentiated capabilities for immunoglobulin biosynthesis of hybrid cells induced between immunoglobulin-producing and non-producing parental cells. Hybridization of human lymphoblasts proved to be a particularly difficult problem which required more effort than initially expected. Thus, interspecific hybrids were obtained relatively late in these studies and only a few hybrids were immunologically characterized.

Initial observations indicated that β-propiolactone-inactivated Sendai virus was effective at stimulating fusion and hybridization of mouse and hamster cells, but was apparently ineffective for inducing hybridization of human lymphoblasts and rodent cells. A stimulation of cell fusion was obtained following Sendai treatment, but cell hybrids were not obtained in early experiments. Attempts to improve cell fusion and hybridization frequencies by varying pH of the medium indicated that fusion, but not hybridization, was slightly enhanced between pH 7.4-7.6. Increasing the concentration of Sendai virus above 2500 HAU/ml slightly decreased the percentages of multinucleated cells observed. The first successful isolation of lymphoblast x rodent cell hybrids was obtained by increasing the ratio of Lymphoblast:rodent cells from 1:1 to as high as 10:1. A maximum frequency

of 1-2 x 10^{-6} hybridization, however, was obtained irrespective of the addition or absence of Sendai virus.

Alternative experiments on non-viral methods for cell fusion indicated that treatment of lymphoblast and fibroblast cells in the presence of trypsin followed by co-centrifugation was nearly as effective as Sendai virus for induction of multinucleated cells. Hybridization frequencies obtained with this non-viral method, however, were 10-13 fold higher than those obtained with Sendai virus. Cell fusion was reduced if co-centrifugation of parental cells was omitted, or if trypsin action was inhibited with fetal calf serum prior to centrifugation. Hybridization frequencies increased with increasing trypsin concentration and reached a maximum of 5.5 x 10^{-5} for MGK8E x 3T3c2 cells treated with either 0.25% Bacto-trypsin or 0.05 mg/ml crystallized trypsin prior to co-centrifugation. Results indicated that both trypsin treatment and co-centrifugation of cells were required for maximum hybridization frequencies. However, co-centrifugation was of primary importance, since hybrid frequencies were significantly enhanced without trypsin treatment when unchilled cells were co-centrifuged at room temperature. Our studies indicated that cell fusion induced by Sendai virus or trypsin-centrifugation was dependent upon the particular pair of lymphoblast and fibroblast cell lines studied. Hybridization of the T-5-1 lymphoblast line, characterized by high spontaneous frequencies, was inhibited following either trypsin or Sendai treatment.

Characterization of various lymphoblast x mouse fibroblast hybrid cell populations isolated in this study was performed with various immunological methods. Immunofluorescence analyses indicated that lymphoblast MGK8E was incapable of immunoglobulin synthesis and that heavy or light chain synthetic capability was not activated in interspecific hybrids. Quantitative immunological analyses indicated that capability for synthesis of IgM heavy chains by lymphoblast line LPM 10-20 was not retained in the HLPM-1 hybrid of LPM 10-20 and mouse 3T3c2 cells. Further characterization of hybrid cell capabilities will be possible as a result of refinement of immunological tests and the successful isolation of a number of different hybrids during the latter portion of this study.