

**THE USE OF FLOW MICROFLUORIMETRY
IN THE ANALYSIS OF THE PHENOTYPE EXPRESSION
OF MOUSE HISTOCOMPATIBILITY ANTIGENS**

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Quantitation of the expression of cell surface antigens has hitherto been limited to analysis by either cytotoxicity tests or radioimmune assays (5, 15). We report here the use of a new methodology

to analyze and quantitate the expression of mouse histocompatibility antigens (H-2 locus) in hybrid clones and parental cell types.

The binding of fluorescein-tagged antibody is measured on a cell-to-cell basis in large viable cell populations using flow microfluorimetric techniques. These techniques have been used to measure hapten and immunoglobulin binding to lymphocyte populations (8, 9, 14). However, this is the first report in which these techniques have been used to examine the expression of the H-2 locus.

The advantage of this approach is twofold: first, a large and statistically significant sample population may be analyzed one cell at a time, thus revealing the fine detail of heterogeneity in the expression of cell surface markers within a population. Second, as has been demonstrated for analysis of specific components of the immune system, this method does permit fluorescence-activated sorting of cell types according to their different surface populations (8, 9, 14).

MATERIALS AND METHODS

Intraspecific somatic cell hybrids were produced by Sendai virus-mediated fusion of clone-1-D cells (4) originally derived from C3H mice (H-2^k) with peripheral white blood cells derived from Balb/c (H-2^d) mice (10).

Clone-1-D cells are resistant to 30 μ g/ml BrdU and do not survive in HAT medium. The peripheral leukocytes do not attach to the glass or survive for long periods in vitro. Thus, only the hybrid cells survive and grow. Colonies were transferred to T-75 Falcon flasks and allowed to grow until they reached a confluent monolayer. Approximately 6 mo elapsed between the time of fusion and these analyses. Two colonies (364-2 col 2 and 364-4 col 1) were used in these investigations.

Cell Lines

For control purposes two Balb/c-derived cell lines were used. One was a suspension cell from a mouse plasmacytoma and the other a glass-attached cell line derived from a Balb/c embryo at 20 days of gestation.

Balb/c Anti-C3H Serum

C3H spleen cells were injected six to eight times intraperitoneally at biweekly intervals (10^6 cells per Balb/c mouse per injection). Mice were bled by cardiac puncture 10 days after the last challenge and sera pooled. Antiserum was absorbed with 10^7 plasmacytoma cells of Balb/c origin. Specificity of absorbed serum was confirmed by indirect immunofluorescence assay, using cell lines and fresh spleen cells of C3H and Balb/c origin.

C3H Anti-Balb/c Serum

The procedure described above was used except that Balb/c spleen cells were injected into C3H/HeJ mice and

the resultant pooled serum was absorbed with clone-1-D cells.

NIH Typing Sera

The following antisera were obtained from the National Institutes of Health: AS-382 detecting private specificity 31 of the H-2^d locus (K-region specificity), C-1 detecting specificity 1 of the H-2^k locus (D-region specificity), and C-3b detecting public specificity 3 expressed on both H-2^d and H-2^k cells (D-region specificity).

Rabbit Immunoglobulin G (IgG)

Anti-Mouse IgG Fluorescein Isothiocyanate (FITC) Conjugate

This product was purchased from Cappel Laboratories, Downingtown, Pa. and used at a 1:10 dilution in Dulbecco's phosphate-buffered saline (PBS) for fluorescence labeling of cells after pretreatment with different murine sera.

Iodination of Antisera (Antiglobulin)

Rabbit IgG anti-mouse IgG FITC conjugates were iodinated using a modification of the chloramine-T method (7).

Measurement of Bound Antibody by

Indirect Labeling Assays

IMMUNOFLUORESCENCE: Single cell suspensions were used for these assays (12). $2-5 \times 10^6$ cells were incubated with antisera 1:10 dilution for 1 h at 37°C. Cells were washed three times in minimal essential medium (MEM) containing 5% fetal calf serum. They were resuspended and incubated with FITC-tagged rabbit IgG anti-mouse IgG (Cappel Laboratories) 1:10 dilution for 1 h at 37°C, then washed, and resuspended in MEM for analysis in the flow microfluorimeter (FMF). Smaller cell numbers (5×10^4 – 5×10^5) were used for UV microscopy; they were finally resuspended in PBS and glycerol (1:1).

RADIOIMMUNE ASSAY: Serum binding was measured on single cell suspensions as in Goldstein et al. (5).

DNA Determination

Cell suspensions were stained with 0.2% acriflavine using a modification of the technique described by Kraemer et al. (12) and analyzed in the FMF.

Flow Microfluorimetry (FMF)

The machine used in these experiments is one currently in use at the Biomedical Division, Lawrence Livermore Laboratories, Livermore, Calif. The basic design and description have been detailed elsewhere (6, 17, 18).

The data are displayed as a frequency distribution; the

abscissa (channel number) indicates the amount of fluorescence detected and the ordinate indicates the relative number of cells having that amount of fluorescence. The frequency distribution thus reflects the amount of fluorescence detected on each cell in the population. Data are expressed either as a frequency distribution or, for comparative purposes, as the channel number in which the modal number of fluorescent cells lies. Implicit in this analysis is the direct relationship between the amount of fluorescence detected and either the DNA content or the amount of fluorescent antibody bound.

In each experiment, calibration of the system was effected by measuring scattered light from 2.02- μ m spheres using a Neutral Density Filter, OD = 1.0. The system was recalibrated before each experiment and after every tenth sample within an experiment.

Fluorescence Microscopy

All observations were made with a Leitz Ortholux microscope utilizing a HBO 200-W mercury bulb as the fluorescent light source. The filters used were 2 mm OGI + 2 mm BG 12 for excitation and K530 for suppression. Cells were scored by two observers immediately and again after refrigeration overnight (1, 11).

Volume Determinations

Cell volume was determined using Coulter Counter model ZBI (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.) interfaced with a 400-channel analyzer.

RESULTS AND DISCUSSION

We have demonstrated the hybrid nature of clones 364-2 col 2 and 364-4 col 1 by their growth in selective medium, by karyotype analysis, and by examining their reactivity with NIH H-2 typing sera directed towards specificities of the parental cells.

Chromosome counts reveal a mean number of 57 (range 54–59) chromosomes in clone-1-D parental cell line, including 11 biarmed chromosomes, one of which is a dicentric. These biarmed chromosomes are on the whole maintained in the two hybrid clones. Each clone contains a mean of 82 (range 72–98) chromosomes, and they appear to be derived by fusion of one clone-1-D cell and one normal mouse white blood cell ($2n = 40$). There has been a loss of approximately 15 telocentric chromosomes between time of fusion and chromosomal analysis.

Fig. 1 shows results of staining these different cell lines with acriflavine to determine their DNA content. Using the modal frequency of the DNA content for G_1 cells, it is evident that both hybrid

clones have more DNA than the clone-1-D parental cells, thus corroborating the difference in the chromosome counts. Coulter Counter determinations indicate that both hybrid clone populations have a larger volume, approximately twice that of the parental cell type clone-1-D (see Fig. 1 and Table II). However, there is no meaningful difference in volume between the cell populations of the different hybrid clones.

FMF analyses of the binding of the different antisera and fluoresceinated rabbit IgG anti-mouse IgG on clone-1-D, 364-2 col 2, and 364-4 col 1 cells are shown in Fig. 1. These frequency distributions appear to be Gaussian with a right-handed skew and are very similar in this respect to the volume spectra derived from the Coulter Counter. This correlation strongly suggests that for these cell lines the amount of antibody bound is proportional either to volume or some parameter related to volume such as surface area.

Comparison of the frequency distributions in Fig. 1 indicates differential binding for each staining reaction among all three cell lines investigated. In Table I, we have combined the results of several experiments; in addition, we have subtracted the modal frequency obtained when the cell populations are reacted with FITC-tagged rabbit IgG anti-murine IgG alone. When this is done, the differential binding of both anti-Balb/c and anti-C3H sera to the different cell lines is greatly minimized.

To confirm these results, radioimmune assays using the same antiglobulin tagged with 125 I were carried out. Saturating conditions were used to maximize the binding capacity of the different reagents. Results are in Table II; they confirm the

TABLE I
Measurements of Volume and Fluorescent Antibody Staining for Two Hybrid Clones and One Parental Cell Line

Cell line	Antisera		Volume
	Anti-Balb/c	Anti-C3H	
Clone-1-D (3)	0	28 [26–32]	15 [13–17]
364-2 col 2 (3)	18 [17–22]	34 [32–40]	30 [29–33]
364-4 col 1 (9)	9 [5–18]	26 [15–35]	29 [27–32]

Measurements are expressed according to the modal channel number. For the fluorescent antibody-staining reaction, the modal channel number for rabbit anti-mouse IgG has been subtracted. The numbers in parentheses represent the number of experiments, in brackets the range of values.

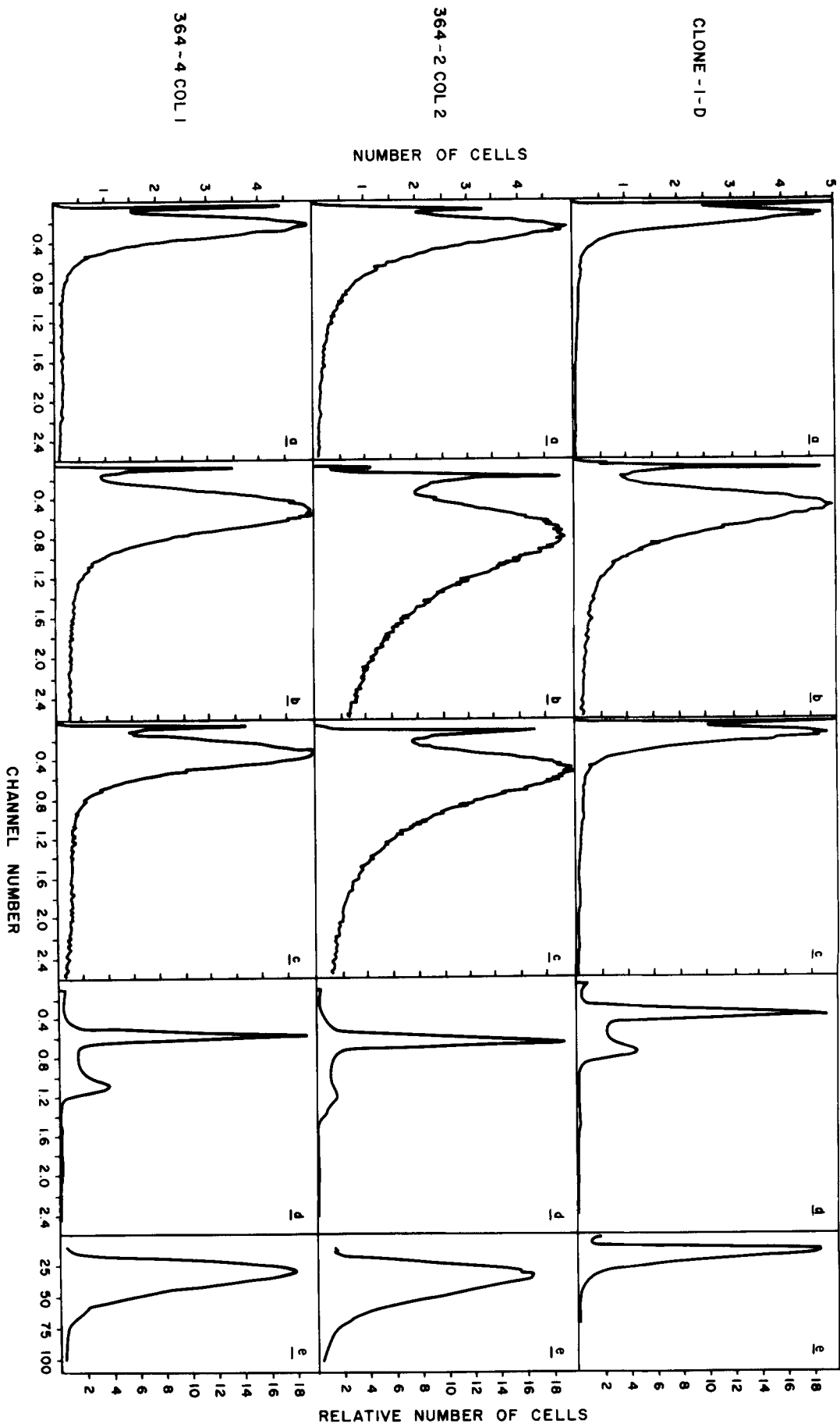


TABLE II
Indirect Labeling of Cell Surfaces with ¹²⁵I-Tagged
Antiglobulin after Reaction with Murine Sera

Cell line	No serum	Murine sera		
		Normal serum 1:10	C3H anti-Balb/c 1:10	Balb/c anti-C3H 1:10
Balb/c fetal cell line	306	221	769	119
Clone-1-D	180	222	270	1,222
364-2 col 2	564	335	1,385	1,703
364-4 col 1	381	262	1,098	1,452

Data presented as average counts per minute per 10⁶ cells from triplicate samples. The coefficient of variation for the triplicate samples is less than 10%. The counts derived from nonspecific binding with no serum have been subtracted from the other three groups.

Antiglobulin: Rabbit IgG anti-mouse IgG FITC-tagged and ¹²⁵I tagged. Input counts: 20,761.

data derived from FMF analyses. (a) 364-2 col 2 binds more ¹²⁵I-tagged antiglobulin than 364-4 col 1, both alone and in the presence of normal mouse serum. 364-4 col 1 binds more than clone-1-D in both situations. (b) There are small differences in the binding to each of the hybrid clones using either anti-Balb/c or anti-C3H serum. These differences remain even after accounting for the differential binding to these cells of both normal mouse serum and the anti-murine IgG.

The most significant finding arises from a comparison of antibody binding and volume. clone-1-D and the hybrid clones bind similar amounts of Balb/c anti-C3H serum. clone-1-D is half the volume of the clones, and hence has a higher ratio of antibody bound to volume. This indicates a higher antigen per unit volume for clone-1-D cells, and strongly suggests a higher density of antigenic sites. Rubio (15) has derived similar evidence using cytotoxicity tests for H-2 alloantigens in parental and hybrid clones.

Single parameter analyses have measured several parameters at the level of the single cell (2, 12, 13). The results in this report indicate that simultaneous measurements of several parameters, as

afforded by FMF, may provide an even more powerful analytical approach (see also 3, 16). Such analyses of density of cell surface antigen expression may be critical in an understanding of their biological significance in cell-to-cell interaction. In addition, information derived from these comparative analytical studies may prove essential in the design of experiments for electronic cell sorting of specific cell types.

SUMMARY

We have analyzed the expression of histocompatibility antigens of intraspecific mouse somatic cell hybrid clones and parental cells. FMF has been used to measure the binding of fluorescein-labeled antibody to the different cell types. Correlation of volume determinations and antibody binding indicate that there are differences in the density of antigen expression between the parental cell line and the hybrid clones.

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FIGURE 1 Frequency distributions of antibody binding, DNA content, and volume of two hybrid clones and one parental cell type. (a) Rabbit IgG anti-mouse IgG FITC conjugated. (b) Balb/c anti-C3H + rabbit IgG anti-mouse IgG FITC conjugated. (c) C3H anti-Balb/c + rabbit IgG anti-mouse IgG FITC conjugated. (d) DNA content - acriflavin stain. (e) Volume.

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