Proc. Natl. Acad. Sci. USA
Vol. 78, No. 1, pp. 564–568, January 1981
Immunology

Expression of μ and γ immunoglobulin heavy chains in different cells of a cloned mouse lymphoid line

(hybridomas/immunofluorescence/gel electrophoresis/enzyme-linked immunosorbent assay/karyotype)

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Communicated by Niels Kaj Jerne, October 13, 1980

ABSTRACT **A cloned cell line derived from mouse bone marrow and transformed by Abelson virus is shown to synthesize two different heavy chains, μ and γ_{2B}, *in vitro*. This characteristic is stable because it persists upon subcloning. Although most of the immunoglobulin-synthesizing cells produce either μ or γ_{2B} heavy chains, a few cells contain both heavy chains, suggesting immunoglobulin class switching. Karyotypes show a complement of 41 chromosomes. Two copies of chromosome 12, to which immunoglobulin heavy chain structural genes have been assigned, were found. No light chain was found in either the μ- or the γ_{2B}-producing cells. However, fusion of the cell line with a myeloma that synthesizes neither heavy nor light chains caused expression of κ light chain in the hybridomas synthesizing μ chain. No light chain could be detected in the hybridomas synthesizing γ_{2B} heavy chain.**

During the immune response, the predominant antibody heavy-chain isotype changes from the μ class, in the primary response, to the various γ classes and the α class in the secondary response. The cellular basis for this phenomenon, termed heavy-chain class switching, has been clarified by a number of experiments. It is thought that the precursors of cells synthesizing any class of immunoglobulin (Ig) initially express membrane IgM because chronic treatment *in vivo* with antibodies to μ chain results in panhypoglobulinemia (reviewed in refs. 1 and 2). Antiserum to μ chain also suppresses the production of all Ig classes by B lymphocytes stimulated with antigen or mitogen *in vitro* (3, 4). That a single IgM-synthesizing cell can give rise to daughter cells synthesizing IgG was shown directly by micromanipulation of single cells after stimulation by mitogen (5). Several mechanisms have been proposed to explain the heavy-chain class switch on a molecular basis. Recent evidence suggests an excision model, as originally proposed by Kabat (6), operating at the DNA level. For the switch, the previously transcribed gene for the constant region of μ chain would be looped out and excised, bringing a different gene for the constant region of the heavy chain (C_H) into proximity with the original gene for the variable region of the heavy chain (V_H) to form an active transcriptional unit (7–12). However, these studies have relied on analysis of DNA from independently derived myelomas synthesizing the different Ig isotypes. Such myelomas have abnormal karyotypes, have undergone laboratory selection for Ig secretion for many years, and, in addition, represent the final stages of B-cell differentiation. Thus, the deletion of the gene for the μ constant region seen in γ-producing myelomas could be a stabilizing rather than a necessary event for the heavy-chain switch.

Indeed, it is not clear at what stage of B-cell differentiation the class switch occurs: mitogen-stimulated B cells begin to synthesize both μ and γ chains within a few hours after addition of mitogen, indicating that the switch at the DNA level, but not its expression, has already occurred before blastogenesis (13, 14). That class switching may be operative even earlier in differentiation, in the precursor of B cells [the pre-B cell (15)], has been suggested by studies of acute lymphoblastic leukemia in humans. A small percentage of leukemic cells with pre-B-cell characteristics synthesizes both μ and γ chains (16).

A cell line able to undergo heavy-chain class switching *in vitro* would be useful for clarifying the molecular basis of the switch and for understanding the regulatory processes involved. In this report we describe a cell line, transformed by Abelson murine leukemia virus, in which cells synthesizing μ and other cells synthesizing γ_{2B} can be found.

MATERIALS AND METHODS

Cell Lines. The cell line transformed by Abelson murine leukemia virus, 18-81, was kindly provided by N. Rosenberg and D. Baltimore. It is a subclone of 18-8, a cell line derived *in vitro* as a transformed focus from BALB/c mouse bone marrow. Some of the characteristics of 18-8 and 18-81 have been described (17–20).

Isolation and characterization of the non-Ig-producing, hypoxanthine/aminopterin/thymidine (HAT)-sensitive myeloma P3x63 Ag8653 have been described (21). Sp2, a hybridoma producing γ_{2B} and κ, was kindly provided by G. Köhler.

Subcloning. Cell line 18-81 was subcloned either in soft agar or by limiting dilution with syngeneic or xenogeneic (Wistar rat) peritoneal exudate cells as feeder cells. Limiting dilution was performed so that 0.15 cell was seeded per well.

Cell Fusions. Fusions between 18-81 and Ag8653 were performed with polyethylene glycol by the method of Köhler and Milstein (22) as modified by Lemke *et al.* (23). Because the 18-81 cells were not sensitive to HAT, a modification of the method of Wright and Hayflick (24) was used to select against 18-81 cells that had not fused with the myeloma. Immediately prior to fusion, the 18-81 cells were washed twice in RPMI 1640 medium without serum and resuspended at 2×10^6/ml in the same medium. Freshly prepared iodoacetamide (Sigma), 0.2 M in distilled water, was added to give a final concentration of 2 mM. This concentration is 10-fold higher than the minimal dose re-

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; HAT, hypoxanthine/aminopterin/thymidine.

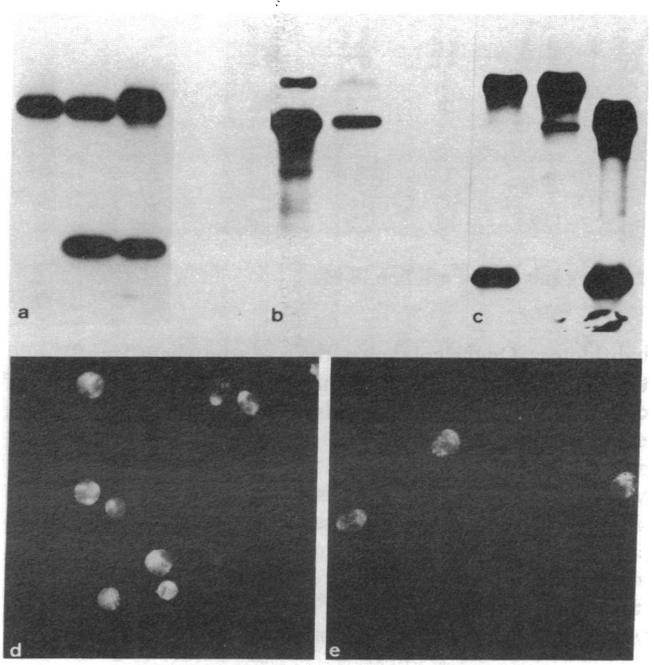

FIG. 1. (*a–c*) Polyacrylamide gel electrophoresis of immunoprecipitated Ig chains synthesized by subclones of cell line 18-81. (*a*) 18-9: Subclone that synthesizes μ chains, but no light chains. Cell lysate precipitated with anti-μ (left lane). 20-25-5: Hybridoma, derived from cell line 18-81 (see text), that synthesizes μ and κ chains. Cell lysate (center lane) and tissue-culture supernatant (right lane) precipitated with anti-μ. (*b*) 12-9: Subclone that synthesizes γ_{2B} chains and no light chains (right lane). 20-34-2: Hybridoma, derived from cell line 18-81, that synthesizes γ_{2B} heavy chain and no light chain (left lane). Both cell lysates were precipitated with anti-μ and anti-γ_2. (*c*) 28-9: Subclone that synthesizes both μ and γ heavy chains—62% μ-positive and 3.5% γ-positive by immunofluorescence. Cell lysate (center lane). Sp2: Hybridoma synthesizing γ_{2B} and κ chains. Secreted material (right lane). 22-25-5: See above. Cell lysate (left lane). All samples were precipitated with anti-μ and anti-γ_2. (*d* and *e*) Cytoplasmic double-immunofluorescent staining of subclone 18-81-289. Fixed cells were stained with rhodamine-coupled anti-μ and fluorescein-coupled anti-γ_2. The same field is shown under selective illumination for rhodamine (*d*) and fluorescein (*e*).

quired to prevent growth of 18-81 cells. The cells were incubated for 25 min at 37°C; then fetal calf serum was added to a final concentration of 25% (vol/vol). The cells were centrifuged and washed twice in medium with 15% fetal calf serum and then once in serum-free medium prior to fusion. For fusion, the ratio of 18-81 cells to myeloma cells was 2:1. HAT was added 24 hr after fusion.

Assay of Hybridomas for Ig Production. Hybridomas were assayed for intracellular and secreted Ig by a modification of the enzyme-linked immunosorbent assay (ELISA) (21, 25). For detection of intracellular Ig, hybridoma cells were added to flexible microtiter plates (M-25 Cooke Laboratory) coated with affinity-purified antibodies to μ, γ_2, or κ chains. The plates were centrifuged, and the supernatant was aspirated. The cell pellet was then lysed *in situ* by addition of 0.5% Nonidet P-40 containing 1 mM phenylmethylsulfonyl fluoride. The assay from this point was identical to that for hybridoma supernatants. Positive results in the ELISA assay were confirmed by immunofluorescence.

Immunofluorescence. The purification and fluorochrome conjugation of goat antibodies to mouse heavy- and light-chain isotypes and methods for immunofluorescent detection of membrane and intracellular Ig have been described (26).

Biosynthetic Labeling of Ig. Cells were cultured at a density of 5×10^6/ml for 4 hr in methionine-free RPMI 1640 medium containing 12% dialyzed fetal calf serum, 50 μM 2-mercaptoethanol, and 100 μCi of [^{35}S]methionine per ml (1 Ci = 3.7×10^{10} becquerels; Amersham Buchler). Another experimental series was done with a tritium-labeled protein hydrolysate (Schwarz/Mann). Cells were washed and lysed with 0.5% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride. Ig was immunoprecipitated from cell lysates or tissue culture supernatants by the *Staphylococcus aureus* technique (27) with the following modifications: (*i*) The buffer for washing the immunoprecipitated Ig–*S. aureus* complexes was 0.5 M NaCl/5 mM Na_2EDTA/50 mM Tris/0.02% NaN_3/0.5% Triton X-100/0.5% deoxycholate/0.1% $NaDodSO_4$/1 mM methionine/1 mg of ovalbumin per ml, pH 8.2. (*ii*) In the cell lines synthesizing γ_{2B}, a clearing precipitation was performed with the protein A-negative *S. aureus* Wood strain. (*iii*) Occasionally, protein A covalently coupled to Sepharose CL-4B was used in place of *S. aureus*. Immunoprecipitated material was reduced with dithiothreitol (50 mM), alkylated with iodoacetamide (100 mM), and analyzed on 10% $NaDodSO_4$/polyacrylamide gels.

Cytogenetic Analysis. Metaphases were prepared and banded with Giemsa/trypsin stain by standard methods (28, 29).

RESULTS

Immunofluorescent Analysis of Cell Line 18-81. Cell line 18-81 has been described by Siden *et al.* (17) as synthesizing μ chain in the absence of light chain. In the same report, the authors indicated that γ-chain synthesis could sometimes be detected immunochemically. By the technique of cytoplasmic immunofluorescence we have consistently found 18-81 cells that produce μ chains and cells that produce γ chains. During 1 year of culture, the frequency of occurrence of μ-producing cells was 5–15%, whereas the γ producers occurred less frequently (0.5–10%). No light chains (κ or λ) were found in either the μ- or the γ-producing cells. Double producers (i.e., single cells synthesizing both μ and γ) were observed with low frequency (0.5% of γ-producing cells also contained μ). With subclass-specific antibodies, we found that the γ chain of 18-81 was of the γ_{2B} subclass. The cell line was negative for γ_1, γ_{2A}, γ_3, and α chains. No surface Ig could be detected.

Subclones. At the time of subcloning, cell line 18-81 had the following characteristics, as determined by cytoplasmic immu-

Table 1. Characteristics of representative subclones of cell line 18-81

Clone	μ, %*	γ, %*	Double producers, %†
28-9	80.8	2.2	3.9
7-3	59.2	1.8	2.6
9-4	59.7	1.8	3.6
18-13	98.3	1.3	4.0
15-6	92.0	<0.2	
11-10	60.1	0.8	
20-2	46.2	0.4	
21-3	2.4	<0.2	
28-1	0.2	6.8	
12-9	<0.01	24.9	<0.2
29-7	<0.01	<0.01	

* Cells were examined for cytoplasmic Ig by immunofluorescence with fluorescein-conjugated anti-μ and rhodamine-conjugated anti-γ_2. Values are derived from at least 500 cells.

† Expressed as percentage of cells positive for γ that were also positive for μ. Values are given only when at least 100 γ-positive cells could be examined for the presence of μ.

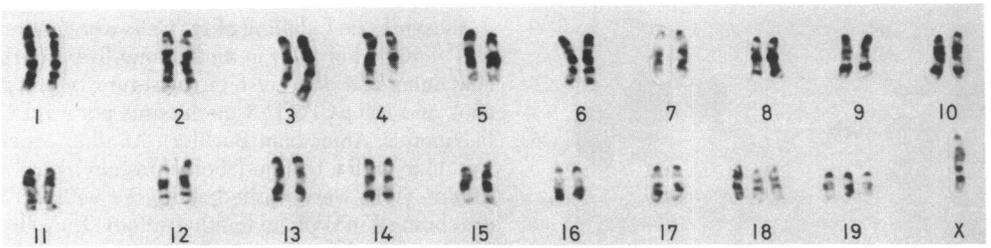

FIG. 2. Karyotype of 28-9, a μ- and γ-synthesizing subclone of cell line 18-81.

nofluorescence: 5% positive for μ, 0.5% positive for γ_{2B}, and 95% negative for Ig. Of the 250 subclones examined, only 3 did not synthesize any Ig when first examined. Three subclones synthesized more γ_{2B} than μ. The remaining 244 subclones contained cells synthesizing μ chain with variable frequency (0.1–90%). Two-thirds of these clones also contained γ_{2B}-producing cells although, in the majority, the frequency was very low ($\approx 10^{-5}$–10^{-3}), and they were not examined further. In 30 subclones, both μ- and γ-positive cells could be found in percentages amenable to analysis. Immunofluorescent characterization of representative subclones can be seen in Fig. 1 *d* and *e* and Table 1. One of the subclones, 29-7, which was negative for Ig production when originally isolated, had 0.5% of cells positive for μ chain after continued passage. Immunochemical analysis confirmed the observation by immunofluorescence. Thus, the 18-9 subclone of 18-81 produces μ chain in the absence of light chain (Fig. 1*a*, left lane). The μ chain has the same apparent molecular weight as the intracellular μ chain (center lane) from a hybridoma derived from cell line 18-81 (see below). The 12-9 subclone produces γ_{2B} heavy chain and no light chain (Fig. 1*b*, right lane). A γ_{2B} chain of identical mobility (Fig. 1*b*, left lane) could also be isolated from a γ_{2B}-producing hybridoma derived from cell line 18-81 (see below). As a standard for γ_{2B} heavy chain, we used the hybridoma Sp2. We could isolate two bands of γ_{2B} heavy chain radioactivity (Fig. 1*c*, right lane) from both cell lysates and supernatants of this cell line, a finding observed previously by others (30). The γ_{2B} heavy chain of 12-9 has the same apparent molecular weight as the smaller of the two γ_{2B} bands of Sp2. The 28-9 subclone synthesizes both μ and γ_{2B} heavy chains (Fig. 1*c*, center lane). Sixty-two percent of the cells were positive for μ chain and 3.5% for γ_{2B} chain, as determined by immunofluorescence. The preponderance of μ-chain synthesis is also apparent in the fluorogram.

Cytogenetic Analysis. Subclone 18-81-28-9 was chosen for analysis because it produces both μ and γ chains (Table 1 and Fig. 1 *c–e*). The chromosome complement is 41, determined on 32 metaphases. As shown in Fig. 2 and by 10 karyotyped metaphases, there is a trisomy of chromosomes 18 and 19. There is only one X chromosome. One homolog of chromosome 3 carries a translocation. Chromosome 12 is present in two copies.

Hybridomas. Of the 55 hybridomas isolated after fusion of 18-81 with Ag8653, 15 synthesized and secreted μ in association with κ light chain. Ten hybridomas synthesized only γ_{2B} heavy chain. Both types of hybridomas synthesized large amounts of Ig and displayed the typical morphology of hybridoma cells. Cytoplasmic immunofluorescence staining is shown for μ chains and γ chains in Fig. 3 *b* and *c*, respectively. Note the heterogeneity of cell size which persists upon subcloning. No light chain was found in the γ_{2B} cells by ELISA or by immunofluorescence. Immunochemical analysis of a hybridoma producing μ and κ and of one synthesizing γ_{2B} but no light chain is seen in Fig. 3. The intracellular μ chain (lanes 4 and 6) has a slightly smaller apparent molecular weight than the μ chain from secreted IgM (lane 5), as observed in other IgM-synthesizing myelomas and hybridomas. On the gel shown, the γ_{2B} heavy chain (lane 2) from the 18-81 hybridoma, 20-34-2, has a molecular weight intermediate between the two γ_{2B} bands of Sp2 (lane 3). Usually, however, its mobility was identical to the smaller γ_{2B} chain (not shown). Another major band, with the same apparent molecular weight as intracellular μ chain, is also immunoprecipitated from 20-34-2. That this is not conventional μ chain was shown by adding 100 μg of purified IgM from the myeloma MOPC 104E to the cell lysate prior to immunoprecipitation. No reduction in the intensity of the band was observed (lane 7). In addition, no μ chain could be detected by immunofluorescence. The extra

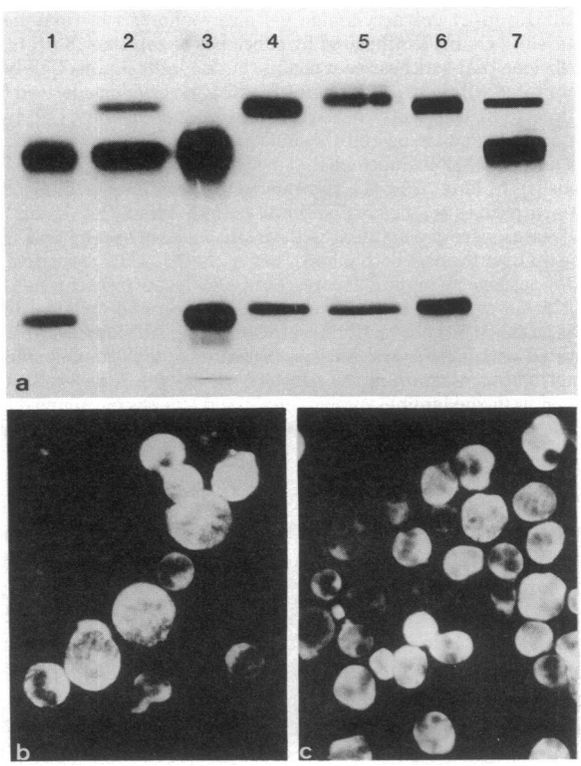

FIG. 3. (*a*) Polyacrylamide gel electrophoresis of immunoprecipitated Ig chains synthesized by hybridomas derived from cell line 18-81. Sp-2: Control hybridoma synthesizing γ_{2B} and κ chains. Cell lysate (lane 1) and tissue culture supernatant (lane 3) precipitated with anti-γ_2. 20-34-2: Hybridoma, derived from 18-81, synthesizing γ_{2B} heavy chain. No light chain was seen when [^{35}S]methionine was used for biosynthetic labeling, a finding also observed with a tritium-labeled protein hydrolysate (not shown). Cell lysate (lane 2) and cell lysate plus 100 μg of unlabeled IgM (lane 7) precipitated with anti-γ_2. 20-25-5: Hybridoma, derived from 18-81, synthesizing μ and κ chains. Cell lysate (lanes 4 and 6) and culture supernatant (lane 5) precipitated with anti-μ. (*b* and *c*) Cytoplasmic immunofluorescent staining of hybridomas derived from cell line 18-81. Magnification is the same as in Fig. 1 *d* and *e*. (*b*) 20-25-5 stained with fluorescein-coupled anti-μ; (*c*) 20-34-2 stained with fluorescein-coupled anti-γ_2.

band is also seen intracellularly in the γ_{2B}-producing subclones of 18-81 (Fig. 1*b*, right lane) and, to a much lesser extent, in cell lysates but not tissue culture supernatants of Sp2 (Fig. 3, lanes 1 and 3, respectively). This higher molecular weight protein could represent the membrane form of γ_{2B} heavy chain because both 20-34-2 and Sp2 express membrane γ chains (not shown). We could find no evidence for secretion of the γ_{2B} heavy chain synthesized by the 20-34-2 hybridoma. The ratio of immunoprecipitable radioactivity in the tissue culture supernatants to immunoprecipitable radioactivity in the cell lysates was 5.7 for the Sp2 control and 0.008 for cell line 20-34-2.

DISCUSSION

We have described a cloned mouse lymphoid cell line that expresses μ and γ Ig heavy chains. Apart from cells in which no Ig heavy chains can be detected, most Ig-positive cells contain either μ or γ_{2B} heavy chain, but we did observe double producers at low frequency.

The information we have obtained from the subclones of 18-81 also provides several clues to the nature of this cell line. Because two-thirds of the 250 subclones of 18-81 contained both μ and γ cells, the ability to synthesize two heavy-chain classes is a normal feature of the behavior of this line *in vitro* and is not due to a single differentiation event that generated γ-producing cells within the parental line. The possibility that the γ-producing cells seen upon subcloning were transformed lymphocytes from the peritoneal exudate feeder cell layer can be excluded because some cells producing μ and γ_{2B} simultaneously were observed and γ-producing cells were seen in clones derived from soft agar or by limiting dilution with rat peritoneal cells as feeder cells. In the latter case, the anti-rat Ig reactivity of the anti-mouse μ and γ_{2B} had been removed by absorption (data not shown).

The Ig-negative cells are able to generate subclones in which both μ- and γ_{2B}-positive cells are found: 95% of cells in the parental 18-81 cell line were negative for Ig, yet 98% of the subclones contained cells synthesizing μ and 65% contained both μ- and γ-producing cells (cloning efficiency, 66%). What is not clear is whether the Ig-negative cells, in fact, do not synthesize Ig or whether they do so at levels not detectable by immunofluorescence. However, we noted a sharp intensity difference between the Ig-positive and Ig-negative cells.

We have identified a novel lymphoid cell type—a γ-producing pre-B cell. This cell, perhaps derived from a μ-producing pre-B cell, lacks surface Ig and light chains and synthesizes only the intracellular form of γ_{2B} chains. This phenotype is stably maintained after fusion with a non-Ig-producing myeloma, as also observed with normal μ-synthesizing pre-B cells (31, 32). Such γ-producing pre-B cells have not been described in fetal liver. However, their low frequency, relative to the already low frequency of pre-B cells synthesizing μ (15), might make a direct demonstration difficult. In the case of human acute lymphocytic leukemia, pre-B cells producing both μ and γ can sometimes be detected (16). One finding that apparently argues against the concept of a pre-B cell that synthesizes γ being derived from one that synthesizes μ is that chronic treatment of mice with anti-μ *in vivo*, if begun at birth, inhibits the development of all isotypes (1, 2) yet has no effect on pre-B cells that produce μ (33). However, such suppression is never complete; measurable levels of IgG can be found in serum.

Because line 18-81 contains single cells producing both μ and γ, it is plausible that the μ-positive cells are giving rise to γ_{2B} cells or vice versa. Thus, we believe that this cell line is an *in vitro* model for Ig heavy-chain class switching. Because both the μ- and γ-producing cells display pre-B-cell characteristics (synthesis of heavy chain in the absence of light chain and no surface deposition of Ig), we suggest that the heavy-chain switch can occur at the pre-B-cell level.

Possible explanations, that μ and γ synthesis is the result of polyploidy of chromosome 12, which carries the structural genes for the Ig heavy chains (34, 35), or that the cell line arose as a fusion between a μ- and a γ-producing cell in bone marrow, were made unlikely by cytogenetic analysis. The chromosome complement of the cell line is 41, with two copies of chromosome 12. The possibility remains that the two heavy chains are synthesized on separate chromosomes.

Perhaps the most intriguing aspect of this cell line is that, although the proposed order of Ig structural genes is μ, γ_3, γ_1, γ_{2B}, γ_{2A}, and α (7), the cells consistently synthesize either μ or γ_{2B}. Thus the commitment to γ_{2B} production is apparently a preprogrammed event in the differentiation of cell line 18-81.

We thank Dr. J. Kearney for generously providing antibodies and M. Tenkhoff for expert help. We appreciate discussions with Dr. C. Steinberg (Basel Institute for Immunology) and with Drs. J. Kearney and M. Cooper (University of Alabama in Birmingham). The initial phase of this study was supported by National Institutes of Health Grants CA 16673 and T32CA09128.

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