Ann. Immunol. (Inst. Pasteur)
1974, 125 C, 271-276

LYMPHOCYTE COMMITMENT TO Ig ALLOTYPE AND CLASS

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The question of whether or not individual lymphoid cell clones are committed to the synthesis of a single polymorphic form of immunoglobulin (Ig) can be most directly pursued by isolating lymphocytes bearing a specific form of membrane Ig and determining whether or not these cells can generate others capable of synthesizing a different Ig molecule. Using a fluorescence-activated cell sorter [1] we have been able to isolate rabbit and mouse lymphocytes which have been membrane stained with fluorescent antibody reagents specific for allotypic or heavy chain determinants for use in studies of commitment.

Rabbit allotype commitment

Because earlier studies had shown that a large proportion of rabbit lymphocytes are capable of binding serum Ig *in vitro* and probably also *in vivo* [3], it was necessary to guarantee that the membrane Ig detected by fluorescent anti-allotype and anti-class reagents was, in fact, synthesized by the cells themselves. In all experiments, therefore, rabbit lymphocytes were incubated for 30 min at 37° C in 0.25 % pronase, a treatment which removes from the cell membranes all the Ig detected by immunofluorescence. The cells were then cultured overnight to allow them to resynthesize the membrane Ig.

To examine the commitment of lymphoid cells to the synthesis of Ig of a single allotype, Peyer's patch lymphocytes from *b5 b9* rabbits heterozygous at the *b* (K chain) locus, after pronase stripping and overnight culture, were membrane stained with rhodamine-labeled anti-b9. They were then passed through the cell sorter which separated the cells into two fractions, one bearing b9 Ig (b9+) and the other with no membrane b9 (b9—). The latter fraction was then stained with fluorescein-labeled anti-

Manuscrit reçu le 3 septembre 1973.

Supported by NIH grants AI 08917, CA 04681 & GM 17367.

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b5 and separated by the cell sorter into a fraction with membrane b5 Ig (b9—, b5+) and a fraction with no detectable *b* allotype (b9—, b5—). To allow the cells to differentiate into antibody forming cells, the cells separated by the machine as well as some of the stained but unseparated cells were put into tissue culture. These microcultures consisted of 2.5×10^5 cells in 0.25 ml RPMI 1640 medium containing 20 % fœtal calf serum (FCS), 2 mM glutamine, and 50 μg/ml gentamycin sulfate solution. To each culture was also added 25 μg/ml pokeweed (PWM) which increases the viable cell recovery after culture and also in some cases the proportion of cells which are synthesizing Ig, without altering the relative proportions of b5 and b9 Ig-synthesizing cells. After four days in culture the cells were harvested. Smears were made, fixed in 95 % ethanol, and stained with fluorescent anti-allotype reagents to determine the relative proportions of cells synthesizing b5 and b9 Ig.

The results of one typical experiment are shown in figure 1. Included

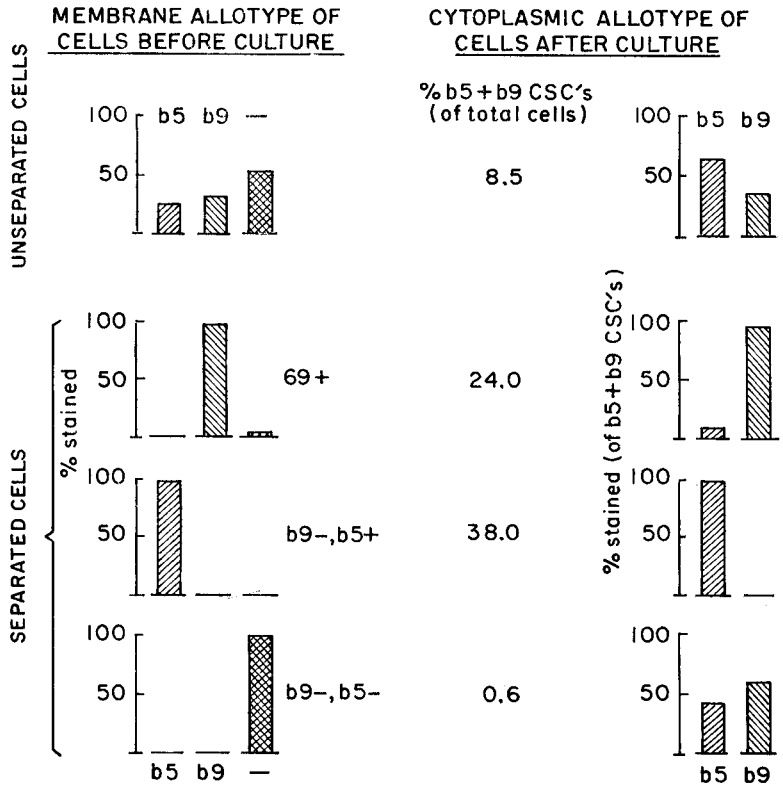

FIG. 1. — *Commitment of rabbit Peyer's patch lymphocytes to Ig allotype.*

Peyer's patch lymphocytes were membrane stained with rhodamine-labeled anti-b9 and passed through the fluorescence cell sorter to separate b9 + cells from b9 — cells. The latter were stained with fluorescein-labeled anti-b5 and re-passed through the cell sorter to isolate b5-bearing cells (b9 —, b5 +) from Ig-negative (b9 —, b5 —) cells. After separation the cells were put into tissue culture; four days later they were harvested and the relative proportions of cytoplasmic staining cells (CSC's) of b5 and b9 allotypes were determined.

in the figure for each cell fraction is the per cent of cells which were membrane stained for b5 or b9 before they were put into culture, the proportion of cells after culture which had cytoplasmic Ig, and the per cent of these which were b5 and b9. Using the cell sorter we obtained fractions which were 97 % b5 and 96 % b9 Ig-bearing, respectively with 1 % or less contamination of cells bearing the wrong allotype. After four days in culture, the b9—, b5+ lymphocyte gave rise to cytoplasmic Ig staining cells (CSC's) which were 100 % b5; the b9+ lymphocytes gave rise to CSC's which were 93 % b9 and 7 % b5. Clearly, then, the vast majority of lymphocytes displaying Ig of one allotype on their membranes are committed to the synthesis of molecules of that allotype when stimulated to differentiate to cells producing large amounts of Ig. Peyer's patch lymphocytes without membrane Ig do not generate large numbers of CSC's (0.6 % of total cells).

In attempting to establish a precursor-product relationship between the type of molecule on a lymphocyte's membrane and the Ig that the cell's antibody-forming descendents will synthesize, it is important to demonstrate that the CSC's present after four days in culture were derived from lymphocytes and not from CSC's present before culture. In most of the experiments there was an absolute increase in the number of CSC's per culture during the culture period; only if the CSC's during culture exhibited much better survival and a higher growth rate than the majority of the cells could they have generated all the CSC's present after four days in culture.

Rabbit H-chain class commitment

Preliminary studies have been done concerning the commitment of rabbit lymphoid cells to the synthesis of a particular heavy chain class. After pronase stripping and overnight culture Peyer's patch and lymph node cells were stained with fluorescein-labeled anti-μ chain and separate in the cell sorter into IgM+ and IgM— fractions. In one such experiment the cell sorter enriched cells bearing IgM from an initial 29 % or up to 93 % for Peyer's patch cells and from 16 to 93 % for lymph node cells. The membrane IgM— cell fractions had less than 1 % IgM+ cells. The cells in these fractions were then stained for IgA, IgG and IgM cytoplasmic Ig, and the per cent of total lymphoid cells which stained for each class are shown in table I. In the Peyer's patch about 12 % of the CSC's with IgM on the membrane had IgA in the cytoplasm, although the majority of IgA CSC's did not membrane stain for IgM. In the lymph node, of the total CSC's with membrane IgM, about 2 % had IgA and 4 % had IgG internally. These results have been confirmed in subsequent experiments and provide evidence that some lymphoid cells are capable of synthesizing Ig of two different classes over a relatively short period of time. Pernis has previously found IgG CSC's with membrane IgM [6], although the cells which were stained in his experiments had not been stripped of membrane Ig and allowed to resynthesize their own Ig.

TABLE I. — **Cytoplasmic immunoglobulin in rabbit lymphoid cells with or without membrane IgM.**

Cells	% Cytoplasmic stained ($\times 10^{-2}$)		
	IgA	IgG	IgM
Peyer's patch			
Unseparated	49	0	70
Membrane IgM +	16	0	112
Membrane IgM −	40	0.4	0
Lymph node			
Unseparated	0.5	3.5	5.7
Membrane IgM +	0.2	0.4	9.2
Membrane IgM −	0	8.9	0

In experiments to be reported elsewhere, cells with IgM on the membrane were put into culture and the class of the Ig made by the CSC's after four days was determined. The data suggest that while IgM-bearing cells give rise predominantly to IgM CSC's, a small proportion of them appear to generate IgA CSC's (Peyer's patch) or both IgA and IgG CSC's (lymph node). This evidence for synthesis of more than one class of Ig by the same lymphoid cell clone has precedent in studies with mice [2, 5, 7] and chickens [4] in which exposure of cells to anti-μ antibodies *in vivo* or *in vitro* blocked the synthesis not only of IgM but also of IgA and IgG, suggesting that lymphocytes bearing IgM are precursors of antibody-forming cells of all classes.

In summary, rabbit lymphocytes from the Peyer's patch are committed to the synthesis of Ig molecules of the allotype and do not switch during differentiation to antibody-forming cells. Although most IgM-bearing lymphocytes probably exhibit the same degree of commitment to heavy chain, some synthesize molecules of different classes and probably have switched.

MOUSE H-CHAIN CLASS COMMITMENT

We also have initiated experiments on class commitment of mouse lymphocytes. The ease with which syngeneic and congenic cell transfers can be carried out successfully in this species will permit precursor-product and possible cell-cell interactions in switching to be studied. In table II are given cytoplasmic staining results for IgM bearing and non-IgM bearing mouse spleen cells from animals primed with KLH many weeks earlier and then boosted several days before sacrifice. It is clear that all the IgM CSC's bear IgM on their membranes and about half the IgG CSC's also have membrane IgM. The other half of the IgG CSC's do not bear IgM. Thus mouse as well as rabbit lymphocytes can synthesize at least two classes of Igs. Whether they stopped synthesis of one before starting synthesis of

TABLE II. — **Cytoplasmic immunoglobulin in mouse spleen cells with or without membrane IgM.**

Spleen cells	% Cytoplasmic stained ($\times 10^{-2}$)			
	IgM	$IgG_1 + IgG_2$	IgG_1	IgG_2
Unseparated	44	12	5	8
Membrane IgM +	48	12	6	9
Membrane IgM −	< 1	12	5	8

the second will be determined in further experiments. Also, although cells with cytoplasmic Ig of two classes were not detected here, we [H. Tacier-Eugster, unpublished results] do have preliminary evidence for such double cytoplasmic stainers at certain stages in the immune response.

SUMMARY

Rabbit lymphocytes from *b5 b9* heterozygotes with surface self-synthesized immunoglobulin of b5 or b9 allotype are committed to produce only that allotype. Thus allelic exclusion is definite when surface Igs are evident. Rabbit and mouse lymphocytes are not irreversibly committed to H-chain class. Cells with surface IgM can have cytoplasmic IgA (rabbit Peyer's patches) or IgG (mouse and rabbit spleen or lymph node).

KEY-WORDS: Allotype, IgA, IgG, IgM, Lymphocyte, Membrane Ig, Cytoplasmic Ig, Allelic exclusion; Mouse, Rabbit, Fluorescent cell separation.

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N. B. — Recent experiments have shown that after 16-20 hours in culture, mouse spleen cells with cytoplasmic IgG do *not* have membrane IgM. Thus, the IgM found on the surface of these IgG cells is either adsorbed *or* is the remnants of IgM produced earlier by cells which have just switched to IgG production.

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