

Antibody production by single, hapten-specific B lymphocytes: An antigen-driven cloning system free of filler or accessory cells

(fluorescein-polymerized flagellin conjugate/interleukins/T cell-replacing factor/B cell mitogens)

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ABSTRACT CBA mouse spleen cells were subjected to hapten affinity fractionation on thin layers of fluorescein (FLU)-gelatin. This procedure yields 97% B cells with varying FLU-binding avidities. One to 30 cells were placed in 10- μ l microcultures without any filler or accessory cells. The T-independent antigen polymerized flagellin coupled to FLU (FLU-POL) was ineffective in stimulating these cells to clonal proliferation or antibody production when used alone. Unpurified preparations rich in interleukins also failed to stimulate the cells. When specific antigen, but not an irrelevant hapten-POL, was combined with the interleukins, clonal proliferation was stimulated and most clones produced anti-FLU antibody-forming cells. The frequency of antibody-forming clones was only slightly lower than that in a system using antigen plus filler cells. In the absence of added interleukins, the mitogens *Escherichia coli* lipopolysaccharide plus dextran sulfate induced equivalent antibody production. However, a higher frequency of clonal proliferation was noted. Added interleukins did not aid these mitogen-driven responses. Such an antigen-dependent cloning system, free of filler and accessory cells, should permit more precise analysis of the respective roles of antigens and interleukins in the physiology of antibody-forming clone formation.

Much of our recent knowledge about B lymphocyte physiology has come from methods that involve cloning by limiting dilution in liquid tissue culture (1-5); in such methods the responding B cell is isolated from most of the effects of the immune network. However, a major disadvantage of these techniques is their dependence on thymic or x-irradiated splenic cells for support of clonal proliferation. Apart from obscuring visualization, these additional cells may produce interleukins and thereby impede the analysis of the effects of intentionally added stimulatory factors. Conditions have been defined whereby single B lymphocytes can be cloned in agar when physically separated from macrophages, which are necessary for their proliferation (6, 7). However, this model allows minimal antibody production (8, 9). Given the current interest in the respective roles of antigens or mitogens and macrophage- or T lymphocyte-derived cytokines in lymphocyte proliferation and differentiation (10-16), a tissue culture system in which B lymphocytes can be stimulated as single cells, free of filler cells, is essential. The various stages in the formation of an antibody-forming cell clone—namely, activation of the small lymphocyte from the G₀ state, mitosis, and differentiation to large-scale antibody synthesis—could then be sequentially studied.

A major step forward in this direction has been taken by Kettman and Wetzel (17-19). They showed that individual murine splenic B cells, stimulated by a mixture of the mitogens *Escherichia coli* lipopolysaccharide (LPS) and dextran sulfate (DXS), proliferated with a cloning efficiency of up to 80%. Only

a minority of the clones produced detectable antibody, and the addition of small numbers of x-irradiated splenic adherent cells, chiefly macrophages, increased this proportion. This prompted us to develop a system in which antigen, acting on affinity-purified antigen-specific B cells (20), rather than mitogens acting on B cells regardless of their specificity, was the primary stimulus, and in which cell-free interleukin-containing media, rather than living macrophages, provided accessory stimulation. We believe such a system will offer better opportunities than mitogen-driven macrophage-containing systems for a progressive dissection of physiological events in antibody formation.

In the present studies, fluorescein (FLU)-specific B cells were prepared from adult mouse spleen through binding to thin layers of FLU-gelatin. The adherent population, 97% surface-immunoglobulin-positive (21), was stimulated with FLU-polymerized flagellin (FLU-POL) and media rich in various interleukins. Single cells generated antibody-forming clones almost as efficiently as in our usual thymus filler cell-containing system.

MATERIALS AND METHODS

Mice and Cell Suspensions. Specific pathogen-free inbred male CBA/CaHWehi mice 8-10 weeks of age were used. Spleen and thymus cell suspensions were prepared as described (3, 22).

FLU Conjugates. FLU-protein conjugates were prepared by allowing the protein to react with fluorescein isothiocyanate in 0.2 M carbonate/hydrogen carbonate buffer as described (23, 24). The FLU-POL used had a substitution ratio of 0.7 mol of FLU per mol of monomeric flagellin, and the FLU-gelatin had 2 molecules of FLU per 100,000 daltons of gelatin.

Preparation of FLU-Specific Splenic B Cells. Spleen cells were fractionated on thin layers of hapten-gelatin as described, using the method of Haas and Layton (20) as modified by ourselves (3, 23, 25). Adherent antigen was removed from the recovered binding population prior to culture by collagenase treatment. This method yields populations of 97% B cells (21), which are over 100-fold enriched for *in vitro* reactivity to hapten-POL conjugates (3, 23, 25).

Preparation of Concanavalin A Spleen Cell Conditioned Medium (CAS) and Macrophage Lysate (LYS). CAS was prepared as described (26). Wistar rat spleen cells were incubated for 2 hr with concanavalin A at 2 μ g/ml, washed, and incubated a further 18 hr in mitogen-free, serum-free medium. The supernatant from the latter incubation is designated CAS. This

Abbreviations: LPS, *Escherichia coli* lipopolysaccharide; DXS, dextran sulfate; FLU, fluorescein; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; POL, polymerized flagellin; LYS, macrophage lysate; CAS, conditioned medium from concanavalin A-stimulated spleen cells; PFC, plaque-forming cell; IL1, interleukin 1; IL2, interleukin 2; TRF, T cell-replacing factor; FLU-SRBC, fluorescein-coupled sheep erythrocytes.

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material is rich in interleukin 2 (IL2) and T cell-replacing factor (TRF) (26, 27) and also contains other cytokines, probably including some interleukin 1 (IL1). A macrophage lysate was prepared essentially as described by Hoffmann (13). Adherent peritoneal exudate cells from *Corynebacterium parvum*-injected mice were stimulated for 4 hr with LPS at 10 $\mu\text{g}/\text{ml}$. After thorough washing, the adherent macrophages were lysed with distilled water. The resulting lysate, designated LYS, was centrifuged, passed through a Millipore filter, divided into aliquots and stored at -20°C . LYS is believed to be rich in IL1 (13). Each batch of CAS and LYS was titrated to its optimal concentration prior to use. CAS was used at between 10% and 30% (vol/vol) and LYS at between 5% and 10% (vol/vol).

Microculture System. Microcultures were set up in 60-well 10- μl Terasaki trays, using culture medium and conditions as described (3, 22, 25). Medium was supplemented with 10% (vol/vol) of a single batch of fetal calf serum (no. 29101002, Flow Laboratories, Stanmore, Australia). Cell suspensions were adjusted such that 10 μl contained a mean of between 1 and 30 hapten-specific cells. Where indicated in the text, 10^5 thymus filler cells, CAS, LYS, or both, LPS (batch 0111:B4, Difco) at 100 $\mu\text{g}/\text{ml}$, and DXS (Sigma) at 10 $\mu\text{g}/\text{ml}$ were added. FLU-POL or 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP)-POL at a final concentration of 0.3 or 1 $\mu\text{g}/\text{ml}$ was used as antigenic stimulus.

Assessment of Clonal Proliferation. Cultures were examined at intervals for up to 7 days, using an inverted phase-contrast microscope at 100-fold magnification, and the number of cells per well and the spatial arrangement of the cells were recorded. In cases in which cultures had been noted to contain only 1 cell by inspection immediately prior to incubation or after 18–20 hr, clone formation was considered positive if >3 cells were observed at subsequent times. In other cases, a well was scored as positive if one or more clusters of >3 blast cells was observed. This method probably overlooked some dispersed clones (19).

Hemolytic Plaque Assay for Anti-FLU Plaque-Forming Cell (PFC) Clones. After 1–7 (usually 3–5) days of culture, trays were washed by flooding with medium. The contents of each well were individually transferred to 96-well, 200- μl capacity, flat-bottomed microtiter plates. Fifty microliters of plaque-revealing mixture [FLU-coupled sheep erythrocytes (FLU-SRBC), complement, and medium in optimal concentrations] was added to each well. After thorough mixing on a mechanical shaker, the trays were centrifuged, incubated (2 hr, 37°C) and wells were scored for the presence or absence of directly hemolytic IgM plaques. The frequency of FLU-specific PFC precursors was determined by Poisson analysis as described in detail by Lefkovits and Waldmann (28). Where appropriate, background clone formation by added thymus filler cells was subtracted. Though the number of plaques per positive clone was not large (commonly 4–10), a frequent observation was the finding of large plaques with a central clump of cells, probably of clonal origin and not dispersed during cell transfer. Our previous experience suggests that most individual cells in such clumps are PFCs (3), and the true number of PFCs per culture was thus higher than the observed number of plaques.

RESULTS

B Lymphocyte Clone Formation After Stimulation of Single Cells with LPS and DXS. The first objective was to confirm the observation (19) that single B lymphocytes could respond to LPS + DXS by proliferation. Accordingly, FLU-gelatin-fractionated cells were dispensed such that cultures came to contain various small numbers of cells. After 18–20 hr of culture, those wells containing only one living cell were identified and observed progressively over the next 5–7 days. With stimulation by LPS

at 100 $\mu\text{g}/\text{ml}$ and DXS at 10 $\mu\text{g}/\text{ml}$, 57 of 179 such cultures (32%) developed into clones. Immunofluorescence studies showed the resultant cells to be surface immunoglobulin positive.

In the course of this work, it became apparent that, at the low cell concentrations operative, the number of cells reaching the bottom of wells as identified by phase-contrast microscopy was lower than that predicted on the basis of hemocytometer counts of the cell suspensions. In four successive experiments, the factor by which the observed number was lower than the predicted number was 2.6 ± 1 . We have not determined whether this is due to early death of cells at extreme dilution or losses due to other factors. We have therefore presented all data on the basis of the mean number of cells believed or predicted to have been placed into a culture well, not on the lower observed number.

Clonal Proliferation and Antibody Production of FLU-Specific B Cells Stimulated by FLU-POL, LYS, and CAS. To determine whether a T lymphocyte-independent antigen, FLU-POL, could substitute for mitogens in causing proliferation of FLU-specific B cells, and to see whether interleukin-rich media could substitute for filler cells and macrophages in promoting B cell differentiation to antibody-forming status, we cultured various numbers of FLU-gelatin-fractionated cells with medium containing FLU-POL, CAS, and LYS. Fig. 1 gives the results of a typical experiment in which 180 replicate cultures were used for each cell dilution. The logarithm of the percent of negative wells was linearly related to the intended input number of cells per well for both clonal proliferation and antibody formation, in accordance with the predictions of the Poisson equation for a "single-hit" phenomenon. The slopes of the regression lines indicated that 1 cell in 18.9 was capable of clonal proliferation, and 1 cell in 25.8 was capable of development into an antibody-forming clone. Had an adjustment been made for the observed numbers of cells actually reaching culture wells, the frequencies would have been considerably higher. Thus, given suitable support by interleukins, single hapten-specific B cells can respond to antigen by proliferation, and, in most cases, by differentiation to PFC.

Kinetics of FLU-POL-, LYS-, and CAS-Stimulated B Cell Response. To determine the kinetics of observed clone formation by single hapten-specific B cells, and of their differentiation to PFC clones, large numbers of replicate cultures were set up and harvested for PFC clone analysis at intervals of 1–7 days after initiation of culture. This study revealed interesting differences between stimulation by FLU-POL + LYS + CAS (Fig. 2) versus LPS + DXS (Fig. 3). In the system driven by antigen + interleukin, both antibody formation and observed clonal proliferation were maximal at day 3, after which the response decreased. In the system driven by mitogen alone (Fig. 3), the overall frequency of clone formation was substantially higher, even without adjustment for cells lost early, and differentiation to PFC status was significantly delayed. By day 3, only 10% of observed mitogen-induced clones had produced PFC, compared to over 30% by day 5. By day 7, even though a substantial proportion of the clones had died, essentially all of the viable clones yielded some PFC. Thus, the mitogenic stimulation favored more extensive proliferation and later cell differentiation, whereas the mixed antigenic-interleukin stimulation favored concomitant proliferation and differentiation. In all the cultures, many dead cells were apparent after day 5, and few cells lived past day 7, in agreement with the findings of Wetzel and Kettman (19).

FLU-POL, LYS, and CAS Stimulation of Single Cells Approaches FLU-POL plus Filler Cells in Inducing Antibody Formation. Table 1 gives the mean results of five experiments

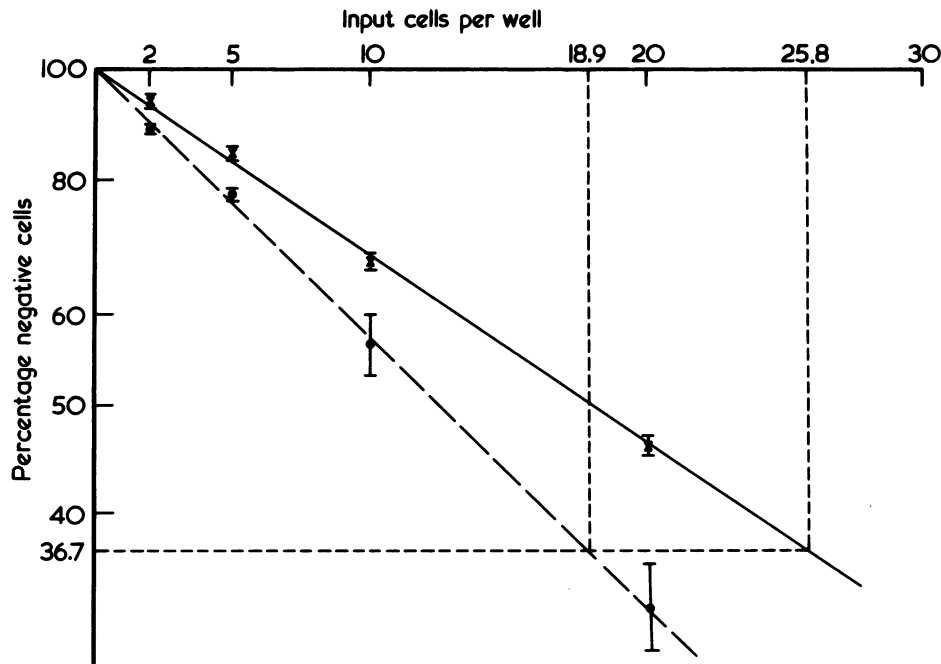


FIG. 1. Linear regression analysis of the frequency of negative culture wells as a function of imputed mean input cell number per well. ●, Clonal proliferation; ×, antibody formation. Vertical bars indicate SEM. FLU-gelatin-fractionated cells were stimulated by FLU-POL, CAS, and LYS. Cultures were harvested at 3 days.

in which a large number of variables in stimulation of clonal proliferation and antibody production were examined. Cultures were assayed at 3 days. Only 1 cell in 3000 divided spontaneously and none formed antibody without stimulation. Interleukin-rich media alone or FLU-POL alone induced only a small number of clones, including PFC clones. The addition of LYS to the antigenic stimulus did not evoke a further response, but FLU-POL + CAS markedly raised both clone formation and antibody production. The further addition of LYS raised the responses only slightly in the experiments shown in Table 1. However, certain batches of CAS showed enhanced activity when LYS was added (data not shown), possibly reflecting various levels of IL1 in different CAS batches. Batches of CAS containing optimal amounts of IL1 would not require the addition of LYS.

To show that the responses to FLU-POL + CAS + LYS were not due solely to some cumulative action of three mitogens, each

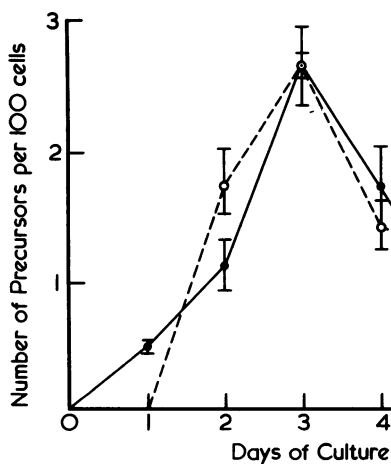


FIG. 2. Kinetics of frequency of clonal proliferation and PFC clone formation after stimulation of FLU-gelatin-fractionated cells by FLU-POL, CAS, and LYS. ○, Clonal proliferation; ●, antibody formation.

at suboptimal concentration (the POL in FLU-POL, the residual small amount of LPS in LYS, and the small residuum of concanavalin A in CAS), the immunogenically distinct but mitogenically equivalent molecule NIP-POL was used with CAS + LYS. This combination was no more effective than CAS + LYS alone in inducing anti-FLU antibody production, proving that specific antigen was indeed operative in promoting proliferation and differentiation.

Antibody-forming cell clones induced in the FLU-POL + CAS + LYS system were relatively smaller when compared to those induced in the standard thymus filler cell system (3), suggesting that stimulation and culture conditions were not ideal. With filler cells, assessment of clonal proliferation is obscured and anti-FLU antibody production was somewhat, but not

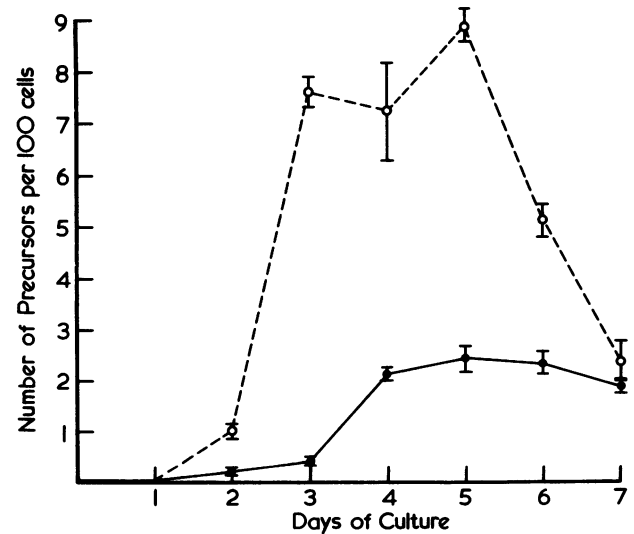


FIG. 3. Kinetics of frequency of clonal proliferation and PFC clone formation by fractionated cells stimulated with LPS and DXS. ○, Clonal proliferation; ●, antibody formation.

Table 1. Frequency of hapten-specific B cells capable of clonal growth or of antibody production in response to various *in vitro* stimuli

Stimuli in medium	% of input cells responding*	
	Formation of visible clone [†]	Anti-FLU antibody production [‡]
None	0.03 ± 0.02	0
CAS	0.29 ± 0.12	0.17 ± 0.06
LYS	0.17 ± 0.10	0.23 ± 0.12
CAS + LYS	0.64 ± 0.21	0.37 ± 0.02
FLU-POL	0.33 ± 0.11	0.83 ± 0.29
FLU-POL + LYS	0.21 ± 0.03	0.28 ± 0.10
FLU-POL + CAS	2.7 ± 0.34	2.3 ± 0.25
FLU-POL + CAS + LYS	3.1 ± 0.37	3.0 ± 0.29
NIP-POL + CAS + LYS	0.51 ± 0.20	0.36 ± 0.11
FLU-POL + fillers [§]	—	5.2 ± 0.72
FLU-POL + CAS + LYS + fillers	—	5.2 ± 0.38

* As determined by Poisson analysis. Each value was derived from study of 120–600 cultures; ± indicates SEM.

[†] A cluster of more than three blast cells appearing viable by phase-contrast morphology.

[‡] As judged by formation of directly hemolytic plaques using FLU-SRBC. Cultures were assayed at 3 days.

[§] Thymus cells at 10⁶ per culture—i.e., 10⁷ per ml—were used because previous work (3) had shown this concentration to be optimal.

markedly, greater (Table 1). The addition of CAS + LYS to the filler cell system did not promote extra antibody production, supporting the possibility that filler cells act, at least in part, by producing interleukins. As our experience with the single cell system grows, the gap in PFC clone frequencies is narrowing, though PFC clone size remains consistently greater when filler cells are used.

CAS + LYS Does Not Promote Differentiation to Antibody Production in the LPS + DXS System. LPS + DXS provokes extensive multiplication but relatively less antibody production among single cells. Kettman and Wetzel (17) have shown that the addition of macrophages increases the percentage of immunoglobulin-producing clones in such cultures. Accordingly, we tested the effects of adding filler cells (containing some macrophages), CAS + LYS, or a combination of CAS + LYS and filler cells, on the mitogen-driven single cell culture system. The results (Table 2) show that filler cells increased the proportion of clones differentiating into PFC. Interestingly, CAS + LYS was slightly inhibitory. In other words, CAS + LYS exerted different effects on single cells stimulated by the mitogen combination than on antigen-stimulated B cells.

Table 2. Frequency of hapten-specific B cells responding to mitogens supplemented by filler cells or interleukins

Stimuli in medium*	% of input cells responding [†]	
	Formation of visible clone	Anti-FLU antibody production
LPS + DXS	13.6 ± 2.6	3.2 ± 0.8
LPS + DXS + fillers	—	8.4 ± 1.2
LPS + DXS + LYS + CAS	10.8 ± 1.2	1.7 ± 0.4
LPS + DXS + fillers + LYS + CAS	—	5.2 ± 0.1

* LPS, 100 µg/ml; DXS, 10 µg/ml; thymus filler cells, 10⁷ per ml; CAS, 10% (vol/vol); LYS, 5% (vol/vol).

[†] Values derived from 180–360 cultures per point. Cultures were assayed after 3 days. ± indicates SEM.

DISCUSSION

Recent work on lymphocyte activation has centered on the respective roles of antigen or mitogen, on the one hand, and antigen-specific or nonspecific, lymphocyte- or macrophage-derived regulatory protein molecules on the other, in the cascade of cellular events involved in an immune response. It is evident that antigen, acting alone on a B cell with surface immunoglobulin receptors reactive with it, cannot promote all the necessary elements of the cascade. Various schemes have been proposed that involve interleukins as cofactors in lymphocyte activation, proliferation, or differentiation. It has become fashionable to state that further progress depends on use of interleukins purified to molecular homogeneity, but this plea, worthy and valid though it may be, is rarely accompanied by a discussion of the difficulties inherent in the purification of proteins acting at extremely low molarity, and identifiable only through complex bioassays. Furthermore, while analysis depends on complex, multicellular tissue culture systems, the possibilities remain that stimulatory signals are not acting directly on the B lymphocyte under consideration, but indirectly on T lymphocytes or macrophages, and that intentionally added interleukins, no matter how pure, are merely supplementing similar or different interleukins manufactured within the complex culture.

For these reasons, we considered the development of a single cell tissue culture cloning system by Kettman and Wetzel (17–19) as a significant achievement. Such a system allows dissection of the influence of defined stimuli on separate events such as blast cell transformation, progressive clonal expansion, and differentiation to PFC status, unimpeded by the unknown effects of the stimuli on other non-B cells in the culture. The first aim of our work, therefore, was to confirm the basic findings of Kettman's group concerning the capacity of LPS + DXS to cause clonal expansion of single B lymphocytes. Our results confirmed that this could be achieved, but our cloning efficiency of 1 cell in 3 of those surviving to 18–20 hr of culture was lower than theirs, namely 80% of B cells. We decided to present all our data on the basis of predicted cell input number, despite the fact that basing our results on 18- to 20-hr counts of cell cultures consistently yielded frequencies 2.6 times higher. This was done to facilitate comparison between results in this paper and our previous work. The cloning efficiencies reported by Kettman and Wetzel (17–19) are based on a different approach, namely the establishment by microscopic observations of the mean number of cells per culture after 12–20 hr. This difference in presentation of results accounts for a major portion of the difference of cloning efficiencies. Furthermore, their studies showed that cloning efficiencies in the LPS + DXS system were dependent on the fetal calf serum batch used (17), and we have not examined this variable. The cloning efficiency is certainly high enough to allow useful consideration of the various stimulatory signals.

Having confirmed the broad thrust of Kettman and Wetzel's (17–19) findings, we introduced some significant variations. Rather than using unfractionated spleen cells, we have concentrated on a population known to be 97% B cells (21), and to bind FLU, though with a range of hapten-binding avidities. This approach allowed study of stimulation by antigens, which would have been impractical with unfractionated spleen cells due to the low frequency of cells reactive to a particular antigen.

Interestingly, specific antigen alone, even when coupled to a multivalent carrier known to be mitogenic in higher concentrations, stimulated the FLU-specific B cells only slightly. However, in the presence of a mixture of interleukins, rich in IL1, IL2, and TRF, the degree of clonal proliferation and differen-

tiation induced was somewhat inferior to that obtained (via unknown effects) by the empirical addition of filler cells. The encouragingly high cloning efficiency of the present antigen-driven system of antibody formation augurs well for further analyses of stimulation and proliferation dependent on purified interleukin sources. We are aware that CAS, rich in TRF, and LYS, rich in IL1, probably contained other interleukins. Indeed, it was not our present purpose to clarify the respective roles of these or other interleukins in B cell activation and differentiation. It was to set the stage for studies in which the separate and sequential effects of semipurified and characterized factors, acting together with authentic antigens rather than unphysiological mitogens, could be examined.

The ability of LPS + DXS to initiate clonal stimulation in a high proportion of B cells in the absence of added interleukins or filler cells, versus the inability of FLU-POL or FLU-LPS in low concentrations (data not shown) acting on FLU-specific B cells to do so, appears contrary to Möller's (29) "one non-specific signal" theory of B lymphocyte activation. The theory postulates that a B cell's Ig receptors function only to focus a mitogen (part of a T-independent antigen or an antigen-containing T cell-derived helper factor) onto the lymphocyte to a high local concentration. Our results suggest that one or more interleukin-mediated signals are comitogenic when acting in concert with antigen, and that somehow the combined use of LPS and DXS overrides the usual need for these ancillary signals.

The greater efficiency of LPS + DXS over interleukin-aided FLU-POL stimulation in promoting clonal proliferation warrants comment. One possibility is that the specific antigen succeeded in stimulating only the higher affinity anti-FLU cells amongst the FLU-specific B cell population, whereas LPS + DXS stimulated cells regardless of their anti-FLU affinity. Alternatively, FLU-POL can stimulate only a subset of the universe of T-independent B lymphocytes (30). Another unresolved issue is why CAS + LYS addition drove most FLU-POL-stimulated clones to antibody formation (Figs. 1 and 2, Table 1), whereas such addition did not accelerate PFC differentiation in mitogen-driven studies (Table 2). Such paradoxes are not surprising when one considers the number of still largely obscure influences affecting the physiology of an antibody-forming clone. Apart from the effects of postulated antigen-specific T lymphocyte-derived helper and suppressor factors, four different sets of stimuli may interact: antigen or mitogen initiating repeated cycles (31) of B cell receptor crosslinking, macrophage-derived IL1 acting as a coactivator, IL2 or some similar T cell-derived B cell growth factor maintaining clonal expansion, and TRF promoting differentiation to PFC status. Mixtures of mitogens could easily subsume two or more of these functions. The availability of affinity-fractionated B cells reactive to specific haptenated immunogens circumvents some of these problems.

The capacity to stimulate them *in vitro* as single cells free of the regulatory influence of other cells opens further perspectives.

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