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Immunology

Three restriction points in the cell cycle of activated murine B lymphocytes

(macrophages/helper T lymphocytes/growth factors)

FRITZ MELCHERS*† AND WALDEMAR LERNHARDT‡

*Basel Institute for Immunology, Basel, Switzerland; and ‡La Jolla Cancer Research Foundation, La Jolla, CA 92037

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ABSTRACT **The cell cycle of activated B lymphocytes was found to be controlled by three restriction points. The first occurs immediately after mitosis and was found to be controlled by the binding of Sepharose-bound, immunoglobulin-specific antibodies to surface membrane-bound immunoglobulin. Exposure to this stimulus as short as 15 min or as long as 36 hr allowed B cells to move into the G_1 phase up to the next restriction point. The second restriction point was observed to be ≈4 hr after mitosis, in the G_1 phase of the cycle and 3–4 hr before the B cells entered S phase, and was found to be controlled by α-type B-cell growth factors produced by the P388D1 macrophage line. A third restriction point occurs in the G_2 phase, 2–4 hr before mitosis, and is apparently controlled by β-type B-cell growth factors that are likely to be produced by helper T lymphocytes.**

DNA replication and mitosis in B lymphocytes are under the control (*i*) of antigen that binds to variable regions of surface membrane-bound immunoglobulin molecules and (*ii*) of two types of B-cell growth factors, α and β, which are produced by macrophages (accessory cells) and by helper T lymphocytes, respectively (for reviews, see refs. 1 and 2). B cells have to be excited from their resting, G_0 state of the cell cycle to become susceptible to the action of α and β factors. This can be achieved in antigen-specific, major histocompatibility complex (MHC)-restricted, T-cell-dependent ways, as well as in polyclonal, MHC-unrestricted, T-cell-independent ways. For the experiments reported in this paper, we activated murine B lymphocytes polyclonally with the mitogen lipopolysaccharide (LPS; ref. 3). The activated B lymphocytes then were synchronized by size-selection using velocity sedimentation (4). This allowed us to investigate in serum-substituted cultures (5, 6) the roles of μ heavy chain-specific monoclonal antibodies (7), as agents acting via surface-bound IgM on B lymphocytes, and of the two types of B-cell growth factors in the control of the cell cycle of these activated B cells. The three interactions—with Ig-specific antibodies, α factors, and β factors—define three restriction points in the B-cell cycle.

MATERIALS AND METHODS

Mice, Cells, and Culture Conditions. Spleen cells from 6- to 12-week-old C57BL/6J *nu/nu* mice (obtained from the Institut für Biologisch-Medizinische Forschung A.G., Füllinsdorf, Switzerland) were prepared and enriched for small, resting cells by velocity sedimentation at unit gravity (4) as described (8). They were cultured at 5×10^5 cells per ml in serum-substituted medium (6) containing 50 μM 2-mercaptoethanol; human transferrin, bovine albumin, and soybean lecithin as serum substitutes; and LPS (*Salmonella abortus equii*, S form, kindly provided by C. Galanos and O. Lüderitz, Max Planck Institut für Immunbiologie, Freiburg i.Br., Federal Republic of Germany) at 25 μg/ml and supplemented with 15% (vol/vol) accessory-cell-conditioned medium (see below) at 37°C in 10% CO_2 atmosphere. After 40–48 hr, the LPS-activated B-cell blasts were collected and washed three times with tissue culture medium at room temperature. They then were separated on the basis of size by a second velocity sedimentation at unit gravity (8). Activated blast cells in 15 fractions (see Fig. 1*a*) were collected by centrifugation at room temperature, washed twice with warm tissue culture medium, and then recultured at 5×10^4 cells per ml in medium containing various stimulatory substances.

Immunoglobulin-Specific Antibodies and Sources of α- and β-Type B-Cell Growth Factors. The monoclonal antibody Ak15, which recognizes the third constant-region domain of the murine immunoglobulin μ heavy chain (7), was purified from tissue culture medium, containing transferrin but not albumin or soybean lipid, in which 2×10^6 Ak15-secreting hybridoma cells per ml had been cultured for 2–3 days. The Ak15 monoclonal antibody-containing medium was dialyzed extensively against 1 mM potassium phosphate (pH 8) and then passed over a DEAE-cellulose column equilibrated with the same buffer, to adsorb Ak15 and transferrin, as well as other molecules that may have been secreted or released by the hybridoma cells. For every 3 liters of conditioned medium, 10 ml of packed DEAE-cellulose was used. The adsorbed proteins were eluted with a linear gradient of 1–250 mM potassium phosphate (pH 8). Ak15 was well-separated from transferrin, as an apparently homogeneous protein fraction, with <1% contaminating proteins detectable as single bands on polyacrylamide gels. After dialysis against the buffer recommended by Pharmacia, Ak15 was coupled under sterile conditions to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) at 2.5 mg of Ak15 protein per ml of swollen Sepharose beads. After saturation of the uncoupled active groups on the Sepharose (done as recommended by the manufacturer), the washed beads were kept as a 10% (vol/vol) suspension in either sterile 0.9% NaCl or sterile culture medium. They were used as a final 1% suspension in all experiments described in *Results*. To remove the beads coated with immunoglobulin-specific antibodies, cultures were resuspended by multiple pipetting. After 5 min, cells could be collected separate from the bead pellet.

Accessory-cell-conditioned medium (as a source of α factors) was prepared with cells of the macrophage line P388D1 (9). The cells were grown to confluence in serum-substituted medium, and then the spent medium and the nonadherent cells were removed and the adherent cells were

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Abbreviations: MHC, major histocompatibility complex; LPS, bacterial lipopolysaccharide.

†To whom reprint requests should be addressed at: Basel Institute for Immunology, Grenzacherstrasse 487, CH-4058 Basel, Switzerland.

cultured with LPS (2 μg/ml) for 48–72 hr. The resulting conditioned medium was filtered through a 0.45-μm Millipore filter and used at 15% in all cultures described in *Results*. The α-factor-containing cultures, therefore, contained as a potential B-cell-activating contamination at most 300 ng of LPS/ml and probably less, since the P388D1 cells would be expected to adsorb some of the LPS. At these low concentrations, LPS will not activate B cells polyclonally to proliferate or to mature (i.e., secrete immunoglobulin) (3). Assays for α factors are described in refs. 9 and 10.

The most convenient sources of helper T-cell-derived B-cell growth factors (β factors) (2) were supernatant media from rat spleen cells stimulated with concanavalin A (5 μg/ml). β factors contained in these supernatants were partially purified by ammonium sulfate precipitation followed by Sephadex G-100 gel filtration (11). A pool of the fractions low in α-factor activities and high in β-factor activities [eluted between 1050 and 1150 ml from a 100 × 5 cm Sephadex G-100 column in 0.9% NaCl/10 mM Hepes, pH 7.3 (see figure 8 of ref. 9)] was used at 5% final concentration as a source of β factors in all cultures described in *Results*. Apart from the low residual α-factor activity, these β-factor preparations are known to contain interleukin 2 and colony-stimulating factors. Assays for β-factor activity are described in ref. 9.

Assays. Incorporation of [*methyl*-^{3}H]thymidine [2 Ci/mmol, 1 μCi per culture (i.e., per 10^4 blast cells originally cultured); 1 Ci = 37 GBq] was allowed to occur for 15 min at 37°C in a 10% CO_2 atmosphere. Incubation was stopped by addition of an equal volume of cold medium containing unlabeled thymidine (100 μg/ml). The cells then were removed from the radioactive medium by centrifugation through a layer of fetal bovine serum, followed by one wash with medium containing nonradioactive thymidine. The incorporation of radioactive thymidine by the B cells was determined as described (8). The relative DNA content per cell was determined by flow cytometry in a cell-sorter. Propidium iodide was used for staining of DNA, and cellular fluorescence was determined in a fashion analogous to the method of Vindelov (12).

Cell concentrations in culture were determined by counting with the aid of a Bürker hemocytometer. Several counts were made with each 200-μl culture. At least 50–100 cells were counted. With 100 cells counted, the accuracy of counting was determined to vary <20% in multiple samplings of the same culture.

RESULTS

Polyclonal Activation, Synchronization, and Restimulation of B Cells. Spleen cells of genetically athymic (T-cell-deficient) C57BL/6J *nu/nu* mice were first enriched for small, resting cells by velocity sedimentation and then activated with the polyclonal B-cell activator LPS. The resulting activated, large cells were separated, again by velocity sedimentation, into 15 fractions that contained cells of different size (Fig. 1*a*). In previous experiments (8), it was found that >90% of all the LPS-activated cells obtained by these procedures are B cells. Cells of fractions 16 and 17 were pooled and subsequently were cultured in the presence or absence of Sepharose-bound immunoglobulin μ-chain-specific antibody (Ak15), α factors, and β factors. Multiple synchronous rounds of division were observed (Fig. 1*b*). Cells divided every 20 hr at 37°C. These results confirm earlier experiments that showed that B-cell clones remain remarkably synchronous for several divisions (13).

Determination of the S Phase in Activated B Cells. To determine the time of S phase within the B-cell cycle in synchronously dividing B cells stimulated by Ak15, α factors, and β factors, incorporation of radioactive thymidine during 15-min periods was measured (Fig. 2*a*). S phase, seen by the increase in thymidine incorporation, appears to occur between hour 8 and hour 16 of the B-cell cycle. Synchrony of the cells was also apparent when the relative DNA content was measured by flow cytometry of propidium iodide-stained cells 2 hr before or after mitosis. Two hours before mitosis, 90% of the population were in S and G_2 phases (15% S, 75% G_2, 10% G_1); 2 hr after mitosis 80% were in G_1 phase (15% G_2, 5% S).

Stimulation by Single Stimuli. In the absence of any stimulus, as well as in the presence of only the immunoglobulin-specific antibody Ak15, only α factors, or only β factors, activated cells completed their cycle but did not initiate a new round of division (Fig. 2). These results confirm earlier observations (10) showing that neither α factors, nor β factors, nor immunoglobulin-specific antibodies alone are sufficient for initiation and completion of the next cell cycle after mitosis.

Synergism of α and β Factors. When α and β factors were added together, B cells divided once more but not further (Fig. 2). They divided at the same time as B cells in the combined presence of Ak15 and α and β factors. These results suggested that immunoglobulin-specific antibodies were needed after each mitosis to render B cells susceptible again to further stimulation by α and β factors. This was tested in the following experiments.

The Role of Surface Membrane-Bound Immunoglobulin. As soon as the B cells had undergone one mitosis in the presence of α and β factors (28 hr of culture), they were washed free of the factors and then exposed to the immunoglobulin μ-chain-specific antibody Ak15 (Fig. 2 *Inset*). In control

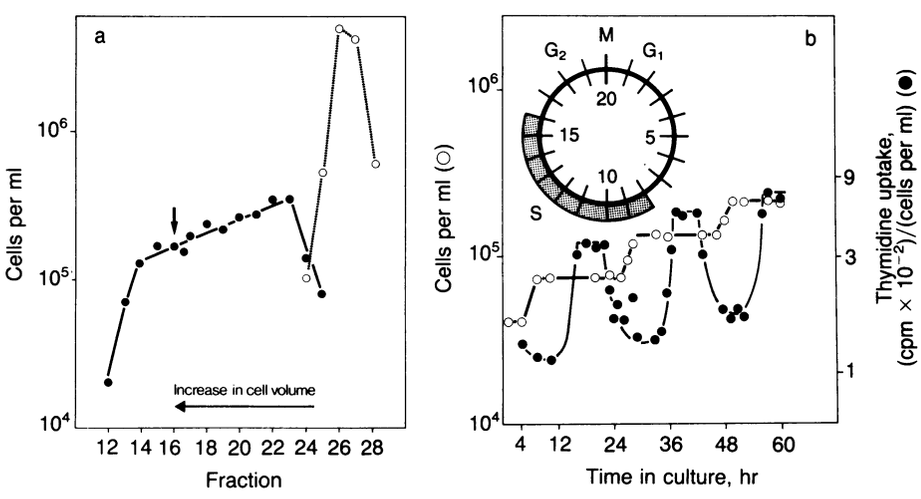

FIG. 1. (*a*) Unit-gravity velocity sedimentation of C57BL/6J *nu/nu* small spleen cells activated for 2 days with LPS. For details, see *Materials and Methods* and ref. 8. The downward arrow indicates the position of those cells in the gradient that were taken for the experiments described in Figs. 3 and 4. ●, Blast cells; ○, nonactivated small cells. (*b*) Thymidine incorporation (●) and cell number (○) in cultures of synchronously dividing B lymphocytes stimulated by Sepharose-bound immunoglobulin-specific antibody Ak15 plus α and β factors. Incorporation was determined after 15-min incubations in the presence of [^{3}H]thymidine. S phase is indicated by the increased uptake of radioactivity observed between 8 and 16 hr after each cell division. (*Inset*) Scheme shows S phase within the 20-hr cycle of B cells.

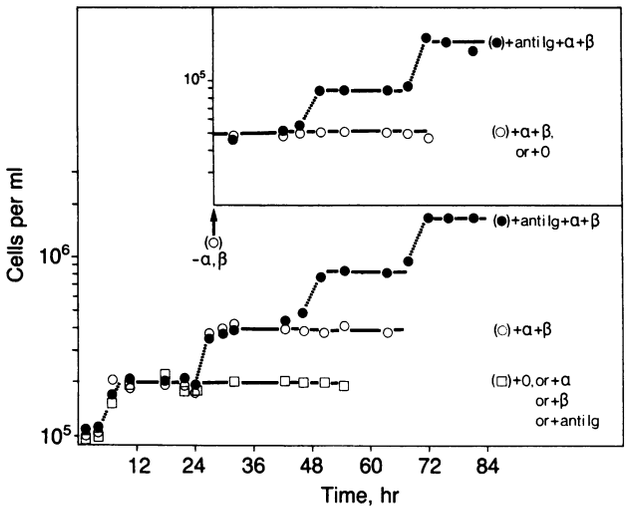

FIG. 2. Growth of LPS-activated, synchronized B cells (fractions 16 and 17, see Fig. 1) in serum-substituted tissue culture medium containing no addition (+0), only α factors, only β factors, or only Sepharose-bound, immunoglobulin-specific antibody Ak15 (anti Ig) (shown for no additions, □); containing α and β factors (○); or containing α and β factors plus Sepharose-bound Ak15 (●). (*Inset*) At 28 hr of incubation (arrow at origin), cells in parallel cultures containing α and β factors were washed to remove these factors and then resuspended in medium with no additions or with α and β factors together (○) or in medium with α and β factors plus Sepharose-bound Ak15 (●).

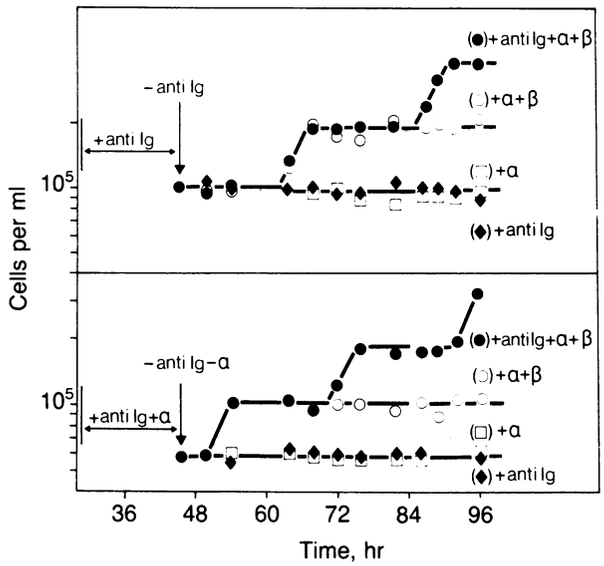

FIG. 3. Growth of LPS-activated, synchronized B cells (fractions 16 and 17, see Fig. 1), grown in the presence of α and β factors for 28 hr and then washed free of factors (see Fig. 2) and exposed between hour 28 and hour 46 to Sepharose-bound, immunoglobulin-specific antibody Ak15 (anti-Ig), either in the presence (*Lower*) or the absence (*Upper*) of α factors. At hour 46, Ak15 (and α factors, if present) was removed, and the cells were washed and resuspended in medium containing Sepharose-bound Ak15 (◆), α factors (□), α and β factors (○), or α and β factors plus Sepharose-bound Ak15 (●).

experiments, α factors, β factors, or α and β factors together were added. As expected from the experiments described above, no further divisions were observed, indicating that it was not the lack of fresh medium or fresh α and/or β factors that limited the capacity of these B cells to divide further (Fig. 2 *Inset*). In another control experiment, cultures to which Ak15 plus α and β factors together were added at 28 hr underwent further synchronous division at nearly the same times of culture (48–52 hr and 68–72 hr) (Fig. 2 *Inset*) as did the cells in those cultures in which Ak15 and α and β factors had been present from the beginning of culture (Fig. 2).

After the first mitosis in the presence of α and β factors, these factors were removed. B cells were then exposed to Ak15 with (Fig. 3 *Lower*) or without (Fig. 3 *Upper*) α factors. No further divisions were observed. This shows that immunoglobulin-specific antibodies, with or without α factors, cannot stimulate B cells after mitosis to complete a new cell cycle with another mitosis. After 18 hr (i.e., at 46 hr of culture in Figs. 1 and 2) specific antibodies (and the α factors) were removed and the B cells were resuspended in medium containing α and β factors either with or without Ak15. Without Ak15, the cells divided once but no more, whereas more than one division was observed in the presence of Ak15. We conclude that exposure to immunoglobulin-specific antibodies, either in the presence of α factors or in their absence, renders recently divided B cells susceptible again to stimulation by α and β factors that allows one further round of division.

Different Restriction Points for the Action of Immunoglobulin-Specific Antibodies, α Factors, and β Factors. Of note are the times at which the B cells divided after they had been exposed first to immunoglobulin-specific antibodies in the presence or absence of α factors and then to α and β factors in the presence or absence of the specific antibodies (Fig. 3). When the recently divided B cells were exposed to Ak15 for 18 hr in the presence of α factors, they divided thereafter at 48–52 hr of culture, at about the same time as B cells exposed continuously to Ak15 and α and β factors. When the recently divided B cells were exposed to Ak15 in the absence of α factors, they divided 12–14 hr after reexposure to α and β factors.

The length of exposure to the μ-chain-specific antibody Ak15, in the presence or absence of α factors, was varied from 15 min to 36 hr. The cells then were exposed to Ak15 and α and β factors to stimulate them through successive rounds of division, and the times at which they divided were observed (Fig. 4).

In the presence of α factors (Fig. 4*a*), exposure to Ak15 could vary from 15 min to 18 hr. In all cases, after reexposure to Ak15 plus α and β factors, the B cells divided at 52–56 hr of culture, close to the time when control cultures divided (Fig. 4*a*, see also Fig. 2). Exposure to Ak15 for 36 hr delayed the division of B cells to 68–72 hr of culture. Division occurred 2–4 hr after addition of Ak15 plus α and β factors, as did those cells that had been exposed to the immunoglobulin-specific antibody for only 18 hr. We conclude from these results that in the presence of immunoglobulin-specific antibodies plus α factors, B cells advance to a point in the cell cycle that is 2–4 hr before mitosis and therefore in the G_2 phase. Exposure to immunoglobulin-specific antibodies can be as short as 15 min to render the B cells competent for the next cell cycle.

In the absence of α factors (Fig. 4*b*), exposure to Ak15 for as little as 15 min kept the time schedule of divisions observed for B cells activated by Ak15 in the presence of α factors. For longer exposure times, longer and longer delays for the division times were observed: for a 4-hr exposure, ≈1 hr; for 10-hr, ≈8 hr; and for 18 hr, ≈16 hr. A delay of 16 hr was also observed for B cells kept for 36 hr in the presence of Ak15 but in the absence of α factors, to a point that is around 16 hr before mitosis and therefore probably in the G_1 phase of the cell cycle.

From these experiments, we conclude that activated B cells have three restriction points that control their cell cycle of 20 hr at 37°C. The first restriction point occurs shortly after mitosis. B cells can pass this point (i.e., enter G_1 phase) when surface membrane-bound immunoglobulin molecules are oc-

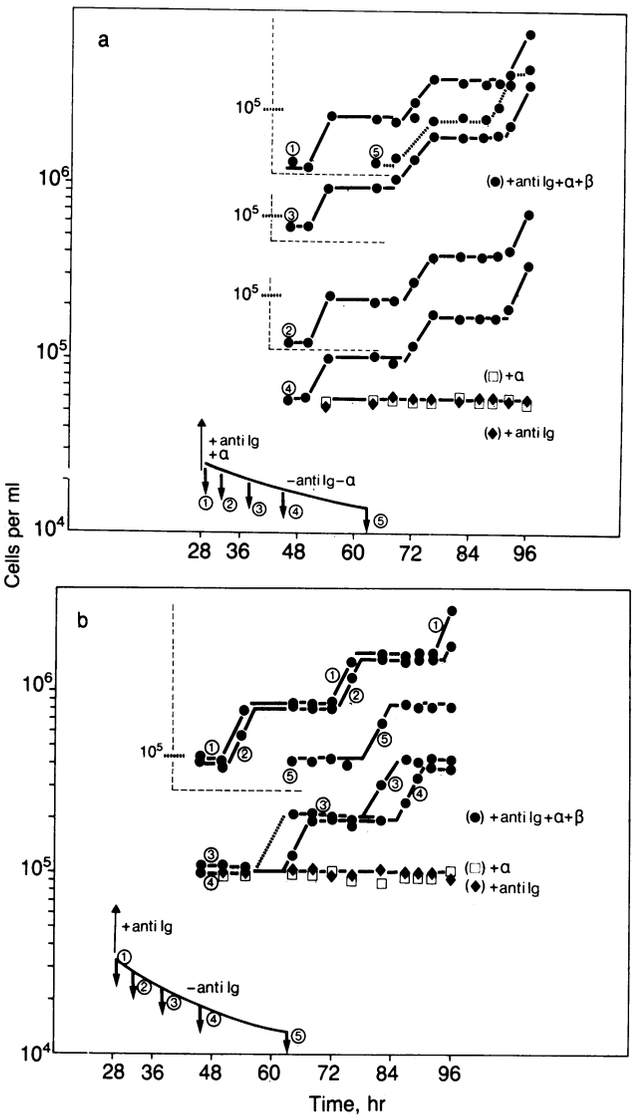

FIG. 4. Growth of LPS-activated, synchronized B cells, grown for 28 hr in α- and β-factor-supplemented medium and then washed free of the factors and exposed (upward arrow) to Sepharose-bound Ak15 (anti-Ig) for 15 min (①), 4 hr (②), 10 hr (③), 18 hr (④), or 36 hr (⑤) in the presence (*a*) or the absence (*b*) of α factors. After these times (downward arrows), the Sepharose-bound Ak15 was removed, and the B cells were washed to remove factors and then resuspended in medium containing α factors (□) or Sepharose-bound Ak15 (♦) (shown only for ④) or containing α and β factors plus Sepharose-bound Ak15 (●). The ordinate (cells per ml) is valid for growth curves 4 in *a* and 3 and 4 in *b*. Growth curves 2, 3, and 1 plus 5 in *a* and 1, 2, and 5 in *b* are plotted relative to the dashed axes (10^5 cells per ml indicated; scale is the same as for the main coordinate system). Counting of cells was begun at 46 hr of incubation; except for growth curves 5.

cupied (in our experiments, by the μ-chain-specific antibody Ak15). The second restriction point occurs ≈4 hr into the cell cycle, about 16 hr before mitosis. B cells can pass this point (enter S phase) when α factors produced by macrophages are present. The third restriction point occurs 2–4 hr before mitosis. B cells require β factors produced by helper T lymphocytes to pass this point.

DISCUSSION

The results presented in this paper could not have been obtained without the development of a serum-substituted, isoosmotic medium (5) that allows B lymphocytes to survive and their responsiveness to growth factors to be retained for days in tissue culture (6) and that excludes possible stimulatory or inhibitory influences of serum components. The experiments also needed to be done without measurable influence of contaminating activated helper T cells (producing β factors) and macrophages or other accessory cells (producing α factors). Spleen cells of specific-pathogen-free, genetically athymic *nu/nu* mice (initially enriched for small, resting cells and, after activation with LPS, again enriched for large, activated blasts) apparently contain so few endogenous contaminating accessory cells or T cells that they did not contribute measurable effects on the factor-dependent cell cycle control of activated B cells.

LPS activates macrophages to α-factor production (10). The level of contaminating, LPS-activatable monocytes that could potentially develop into α-factor-producing macrophages must, however, have been very low since we had to add macrophage (P388D1)-derived α factors in the initial activation of resting cells with LPS to obtain homogeneously asynchronous B-cell blasts (see *Materials and Methods*). Whenever these exogenous α factors were not added, an enrichment for activated cells of relatively small size was observed. Preliminary results show that these cells have undergone initial events of excitation from the resting state (14) and have enlarged but have not yet entered S phase (15, 16).

The removal of LPS from the activated B cells is not easy. If the activated cells are collected and washed at 4°C rather than at room temperature, the B-cell blasts will continue to divide more than once, provided that α factors are present. The one division observed in the absence of β factors with LPS-activated B cells indicates that enough LPS is still present with the cells to stimulate one more mitosis. We have found that the activated B cells undergo several divisions in the presence of LPS when α factors but not β factors are present (unpublished data). LPS must have been present in sufficient concentration, even after the first division of the separated B-cell blasts, to put them in an excited, factor-susceptible state, since α and β factors together stimulated one more mitosis (see *Results*) but no additional ones. This argues for a dual role of LPS, in that it replaces β factors and circumvents the binding step to immunoglobulin.

The occupancy of membrane-bound immunoglobulin on B cells has been identified as one important prerequisite for the excitation of B cells from their resting state (reviewed in ref. 2). T-cell-dependent excitation is often not only antigen-specific but also MHC-restricted (14). Excitation by LPS or by immunoglobulin-specific antibodies, however, is polyclonal and MHC-unrestricted. The experiments presented in the present paper show that polyclonal excitation to α- and β-factor susceptibility by immunogobulin-specific antibodies must occur after each cell division. We expect that this will also be the case for T-cell-dependent, MHC-restricted B-cell proliferation, which we consider to play the major role *in vivo*. We and our co-workers have observed previously that antigen-specific helper T cells, as well as media conditioned by their interaction with antigen and macrophages, stimulate MHC-compatible as well as MHC-incompatible B-cell blasts polyclonally (17, 18). The results presented here suggest that this is true for the next round of division of activated B-cell blasts, but not for more than one round.

The α and β factors we used were not pure and could have been mixtures of molecules. Nevertheless, α and β factors that define separate restriction points in the cell cycle come from different cells that cooperate in immune responses. Our results offer new assay methods for B-cell growth factors: entry into S phase without division of B cells should be used for α factors, and mitosis without DNA synthesis, for β factors. Such assays may help to clear up some of the confusion about how many B-cell growth factors there are

and how α and β factors relate to B-cell growth factors I and II (1, 2).

Different restriction points in the cell cycle have been observed in mammalian cells of other differentiation lineages. Suboptimal growth conditions at high cell density, serum starvation, or limitation of nutrients let some cells enter quiescent states that have often collectively been called G_0 (19–22). There is controversy over whether cells arrested in such G_0 states are actually in identical physiological states. Bearing in mind our findings of different restriction points in the B-cell cycle, it seems possible that different starvation conditions could also arrest cells of other lineages at different points in the cell cycle (23, 24). Our findings raise interesting questions as to whether the G_0 phase of the originally resting B cells from spleen differs from those G_0 phases that are generated in B cells by omission of either immunoglobulin-specific antibodies, or α factors, or β factors.

A restriction point late in G_1 and before S phase appears to exist in several cell types. It has been found to be controlled by essential amino acids, serum, purified multiplication stimulatory activity (25), or purified epidermal growth factor (26, 27). Subsequent experiments (28, 29) have shown that the transition from G_0 to G_1 preceding S phase is under dual control of platelet-derived growth factor and somatomedin C and that induction of competence by platelet-derived growth factor precedes stimulation to progression into S phase mediated by somatomedin C. The restriction point controlled by somatomedin C was found to lie 4 hr prior to S phase (30). This control point of the cell cycle most closely resembles that regulated by macrophage-derived α factors in B cells.

A weak control point of the cell cycle has also been located in the G_2 phase of other cell lineages, where cAMP has been found to arrest V79 cells (31). This control point resembles that controlled by helper-T-cell-derived β factors in B cells. The extended synchrony of B cells for more than four divisions (see Fig. 2) is stunning, particularly since even cloned B-cell lymphomas and myelomas do not display such synchrony (unpublished observations). It is, therefore, possible that synchrony of all B cells in the population is maintained by the secretion of a division-controlling mediator that is made, but also sensed, by all B cells, maybe at a late point in the cell cycle during G_2 phase. This situation could be analogous to that in *Dictyostelium*, which controls synchronized aggregation-competence of cell populations by the secretion and reception of cAMP (32). Why clones of activated B lymphocytes should divide synchronously in the immune system is not clear. If they do so because of the secretion of a soluble mediator, then only those cells that form a cluster would continue to divide *in vivo*. This, in turn, may keep them near to the antigen-presenting macrophages and the cooperating helper T lymphocytes.

It should be remembered that it is at this late point in G_2, when β factors act, that sister chromatids could undergo unequal crossing-over. This mechanism has been inferred in immunoglobulin-heavy chain class switching (33), which, in turn, has been suggested to be controlled by T lymphocytes. A prolonged stay at this point in the cell cycle, effected by temporary lack of T-cell help, followed by rescue with renewed T-cell help, would increase the probability for sister chromatid exchange-mediated class switching. This, in turn, could put the switched B cell under the control of different help.

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