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CELL DIFFERENTIATION IN THE PRESENCE OF CYTOCHALASIN B: STUDIES ON THE "SWITCH" TO IgG SECRETION AFTER POLYCLONAL B CELL ACTIVATION[1]

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Mouse spleen cells were cultured with lipopolysaccharide in conditions that activate both IgM and IgG secretion. Addition of cytochalasin B (CB), an inhibitor of cytokinesis, lead to a high degree of polynucleation, with little effect on Ig secretion. Using cytoplasmic staining with fluorochrome conjugated antisera, we determined the numbers of IgG-containing cells that also contained IgM in their cytoplasm. Such double staining cells were relatively more frequent at early times of the cultures, but at all times single producing cells were in the majority. Addition of CB over the period when the IgG producing cells first appear, lead to a marked increased frequency of double staining, polynucleated cells. This characteristic was stable over a period of at least 42 hr, suggesting that each double staining cell actively synthesized both isotypes. When CB was added after IgG production had started, little increase in the numbers of double staining cells were observed, although polynucleation remained extensive. These data confirm previous findings that the lineage of one cell can produce both IgM and IgG. Furthermore, the results suggest that cells in the process of switching from IgM to IgG go through an asymmetric division leading to one IgM-producing and one IgG-producing daughter cell.

Cytochalasin B (CB)[5] can prevent cytoplasmic division without interfering with other cellular activities, such as nuclear division, protein synthesis, or secretion (1–4). In this report we show that CB provides a suitable means to construct synkaryons, suitable analogs of small cell clones that can help in the study of differentiation within heterogeneous cell populations. We have used this approach to investigate the origin of the IgG-secreting cells that appear in lymphocyte cultures after polyclonal activation by lipopolysaccharide (LPS) (5).

In antigen-stimulated lymphocyte systems it appears that the sequential expression of antibodies of different heavy chain classes occurs within the antigen-specific clone (6–8). The evidence for this depends on the use of the variable region as a clonal marker. Also in lymphocyte cultures that are stimulated by the polyclonal activator, LPS, evidence has been presented that the progenitors of the IgG-synthesizing cells express surface IgM (9–11). By taking advantage of the properties of CB, we provide direct evidence that the nucleus of a single lymphocyte, stimulated by LPS, can give rise to nuclei able to direct synthesis of both IgM and IgG. This is consistent with the recent results by Wabl *et al.* (12), and Anderson *et al.* (13). In addition, the results suggest that differentiation to IgG secretion may involve an asymmetric cell division.

MATERIALS AND METHODS

Mice. C3H/He mice, 5 to 8 weeks old, of both sexes were bred in Department of Radiology, Stanford University Medical Center.

Culture conditions. Spleen cells were cultured at 4×10^5 cells/ml, unless otherwise stated, essentially as described by Kearny and Lawton (5) and Melchers *et al.* (14). The culture medium consisted of RPMI 1640 with addition of 2 mM L-glutamine, 1% penicillin-streptomycin (100 units/ml and 100 μg/ml, respectively), 10 mM HEPES, 15% fetal calf serum [(batch numbers R562122 and C561120) all from Gibco, Grand Island, N. Y.], 5×10^{-5} M 2-mercaptoethanol (Aldrich Chemical Co., Milwaukee, Wis.) and 10 μg/ml LPS [from *E. coli* strain 055:B5, extracted by the Westphaal method, (Difco Laboratories, Detroit, Mich.)]. Micro Test II microplates (No. 3040, Falcon Plastics, Oxnard, Calif.), Tissue Culture Cluster plates (No. 3524, Costar, Cambridge, Mass.) with 0.2 ml/culture, and 1 ml/culture, respectively, or culture flasks (No. 25100, Corning Glass Works, Corning, N. Y.) with 5 to 10 ml/flask were used as culture vessels. CB (Aldrich Chemical Co.) was dissolved in 25% dimethylsulfoxide to a concentration of 100 μg/ml, and was added to cultures at a final concentration of 1 to 5 μg/ml. Higher concentrations lead to more extensive polynucleation, but were also nonspecifically toxic. Control cultures were sometimes treated with an equivalent concentration of dimethylsulfoxide alone, but this did not seem to affect Ig production or secretion.

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[5] Abbreviations used in this paper: CB, cytochalasin B; FITC, fluorescein isothiocyanate; TRITC, tetrarhodamine isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Measurements of Ig-secreting cells. A polyclonal plaque-assay with Staphylococcal Protein A (Pharmacia Fine Chemicals, Piscataway, N. Y.) coupled to sheep erythrocytes was used to measure plaque forming cells (PFC) (15). The following rabbit antisera were used: anti-IgM (anti-MOPC 104E), anti-IgG (7S) (Miles Laboratories, Inc., Elkhart, Ind.), and anti-IgG1 (anti-MOPC 21) (a gift from Dr. F. Melchers, Basel Institute for Immunology). The antisera were tested against a battery of plasmacytomas in the protein A plaque assay. The anti-IgM developed plaques with MOPC 104E ($\mu\lambda$, Ig-6a), TPC 183 ($\mu\kappa$, Ig-6a), HPC 76 ($\mu\kappa$, Ig-6a) and CBPC 112 ($\mu\kappa$, Ig-6b), but not with MOPC 21 ($\gamma_1\kappa$), RPC 5 ($\gamma_{2a}\kappa$), MPC 11 ($\gamma_{2b}\kappa$) or J606 ($\gamma_3\kappa$) as secreting cells. All tumors except CBPC 112 were derived from BALB/c mice, CBPC 112 grows in BAB. 14. The anti-IgG (7S) developed plaques with all the IgG-secreting tumor cells listed above, but not with the IgM-secreting cells. The anti-MOPC 21 was a whole serum, and developed plaques with MOPC 21 (Ig-4a) and MOPC 245T ($\gamma_1\kappa$, Ig-4b, grows in BAB. 14 mice), but not with any other tumors listed, when used at a final concentration of 1/2800. At higher concentrations it developed plaques with RPC 5 as secretor cells, but not with any of the other IgM or IgG-secreting tumor lines. Ten to 60% of the total numbers of viable tumor cells were detected as PFC.

Measurements of Ig-containing cells. Cultured cells were washed in balanced salt solution, and dead cells were removed by Ficoll-Hypaque centrifugation. The samples were cytocentrifuged, fixed, and stored in 95% ethanol at −17°C. The preparations were stained with fluorochrome-conjugated, class-specific antisera directed against IgM, IgG2a or IgG1, as described previously (16). The antisera were raised in goats against murine myeloma proteins and were rendered class specific by removing cross-reacting antibodies on immunoabsorbent columns. Fluorescein isothiocyanate (FITC) conjugates were prepared from the IgG fractions of the antisera (FITC-anti-IgM and FITC-anti-IgG2a). For tetrarhodamine (TRITC) conjugates, specific antibodies were prepared by affinity chromatography (TRITC-anti-IgM and TRITC-anti-IgG1). The fluorescent reagents were found to be specific by staining Sepharose beads coated with different myeloma proteins as well as by cytoplasmic staining of plasmacytomas. The lack of cross-reactivity between the anti-IgM and anti-IgG antisera was also demonstrated by the virtual absence of double staining cells after 9 days of culture with LPS (see Table III).

For the estimation of the total numbers of IgM and IgG2a-containing cells by using the fluorescence microscope, no cut-off filter was used, since this reduced the numbers of detectable stained cells by almost 60% (see below). For the estimation of cells containing both IgM and IgG, cells were first screened for IgG-positive cells. When FITC-anti-IgG2a was used, a SP-540 nm cut-off filter was inserted in order to exclude completely the emission of rhodamine that passes the LP-528 nm barrier filter. Only cells with smoothly stained cytoplasm were accepted as positives. Once a cell was scored as IgG positive, it was determined whether it also contained cytoplasmic IgM by changing the filter sets. The frequency of double staining cells was related to the total numbers of IgG-producing cells. For the CB-treated preparations, only multinucleated cells were considered.

RESULTS

Kinetics of IgM and IgG secretion after LPS activation. When mouse spleen cells are stimulated with LPS in low cell density cultures, both IgM and IgG production occurs (5, 14). In Figure 1, Ig secretion after LPS activation was measured by using a polyclonal plaque assay. Detectable numbers of IgM-secreting cells were found already 1 day after initiation of cultures, and the peak response was obtained Day 3. The IgG-secreting cells, however, stayed below the level of detection up to Day 2, then rose sharply in numbers to reach the peak on approximately Day 5.

Effect of CB on proliferation. In the present study, we have inhibited cytokinesis during the period that IgG-secreting cells first appear and proliferate. We followed the effect of such treatment on a) the number of nuclei per IgM- and IgG-containing cells, b) the switch to IgG secretion, and c) the proportion of IgG-producing cells that also have IgM in their cytoplasm.

Induction of Ig secretion without CB is accompanied by a marked cell proliferation (14, 17) that is most pronounced early after the start of cultures. This is exemplified in Figure 2, where proliferation was measured by counting the numbers of viable cells per culture at different times after LPS activation had started. When CB was added during this growth period, the proliferation of cells ceased within hours, and a decrease in cell numbers was observed (Fig. 2). However, the number of nuclei per cell increased with time as the result of ongoing mitotic activity. In Table I we measured the relative proliferation rate of IgM and IgG2a-producing cells by comparing the degree of polynucleation in IgM and IgG2a-containing cells using the technique of intracytoplasmic staining. As is shown in Table I, both cell types showed a similar degree of polynucleation.

Effect of CB on Ig secretion. We investigated whether Ig secretion was inhibited by CB. In experiment I, Table II, cultures were pulsed with CB for the last 12 hr of culture after which IgM- and IgG-secreting cells were measured. Since cell proliferation ceased due to inhibited cytokinesis (Fig. 2) the numbers of secreting cells per culture were lower in CB treated as compared with control cultures (data not shown). However,

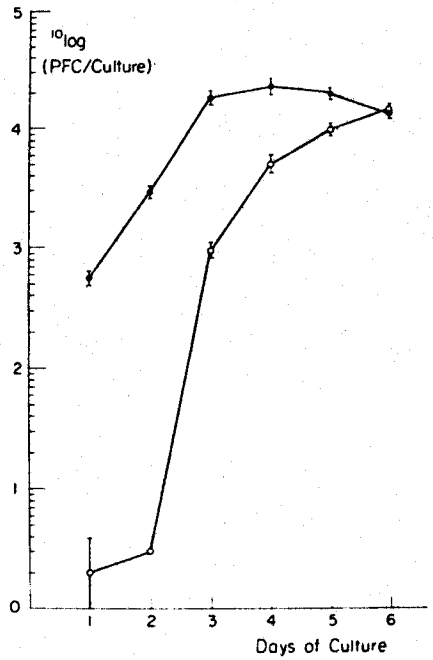

Figure 1. 3×10^5 spleen cells/ml/culture were activated by LPS as described in *Materials and Methods.* Each day, cultures were assayed for the numbers of IgM- and IgG-secreting cells, using the polyclonal plaque assay. Each point represents the geometric mean ± S.D. of triplicates. ●, IgM PFC; ○, IgG PFC.

the numbers of secreting cells per total number of viable cells were comparable in CB-treated and control groups. In experiment II, Table II, CB was added at different time points and remained in the cultures until harvest. The numbers of secreting cells as a fraction of viable cells were lower than in the control groups only after prolonged exposure to CB, possibly due to toxicity of the latter. Higher numbers of secreting cells were occasionally observed, which might indicate that multinucleated cells were more efficiently detected in the plaque assay. Taken together, the data in Table II indicate no marked inhibition of Ig secretion by CB.

Effect of CB on cells containing both IgM and IgG. As shown in Table III, LPS activation led to high frequencies of cells containing IgM or IgG2a in their cytoplasm, as determined by intracytoplasmic staining with fluorochrome-conjugated antisera (10, 11). In agreement with the data presented in Figure 1, IgG2a-producing cells were below the level of detection before Day 3. IgM-producing cells were detected earlier and also peaked earlier than IgG2a-producing cells. With the technique of fluorescent double staining, cells with both IgM and IgG in their cytoplasm were detected in LPS-activated cultures (10, 11). As shown in Table III, these double-staining cells were more frequent early after onset of cultures.

We investigated the effect of CB on the frequency of double containing cells. In untreated cultures, 26% of both the IgG2a and IgG1 containing cells also contained IgM on Day 3. When CB was added on Day 2, and the cultures harvested on Day 3, the fraction of doubles had increased to 83 and 59%, respectively. The fraction of doubles remained high when the CB-treated cultures were harvested after 2 days, i.e., Day 4. How-

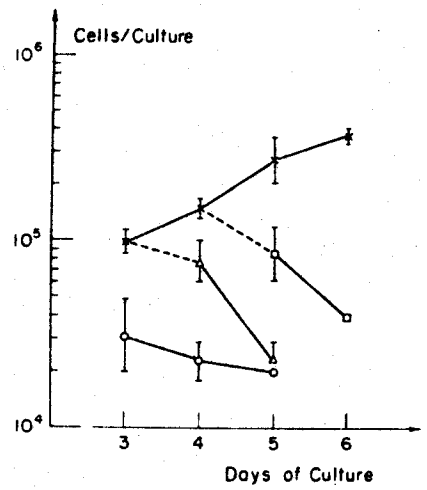

Figure 2. 3×10^5 spleen cells/ml/culture were activated by LPS. One microgram of CB was added at different days, and the numbers of viable cells were determined. ×, no CB added; ○, CB added Day 2; △, CB added day 3; □, CB added Day 4. Each point represents the geometric mean ± S.D. of triplicates.

TABLE I

Percentage of cells containing n nuclei[a]

Length of CB Pulse	Examination of cells Containing	n=	1	2	3	4	5	6	>6	No. of Cells Analyzed
hr										
0	IgM		92	7	1					200
8	IgM		28	51	10	6	4			200
17	IgM		13	42	19	21	3	1		400
17	IgG_{2a}		15	41	15	22	6	1	1	200
25	IgM		15	20	14	28	7	8	7	650
40	IgM		13	20	10	17	7	9	23	550

[a] Estimation of the numbers of nuclei per cell after treatment of LPS-stimulated cells with CB. 3×10^5 spleen cells/ml were cultured. One microgram per milliliter of CB was added on Day 2, and the cells were harvested after indicated times thereafter. Cytocentrifuge smears were prepared, fixed, and stained with TRITC-anti-IgM and FITC-anti-IgG_{2a}. The numbers of nuclei in cells synthesizing either Ig class were estimated by fluorescence microscopy.

TABLE II

PFC/10^4 cells[a]

Treatment	Day 3		Day 4		Day 5		Day 6	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
Experiment I								
No CB	370[b] (±44)	5 (±3)	476 (±83)	19 (±1)	823 (±437)	115 (±11)	605 (±128)	167 (±30)
12 hr CB pulse	491 (±102)	7 (±5)	637 (±292)	18 (±15)	802 (±536)	69 (±37)	n.t.	172 (±82)
Experiment II								
No CB	17 (±9)	≤0.3 (±0.1)	127 (±17)	19 (±6)	386 (±32)	37 (±3)	n.t.	748 (±100)
CB added Day 2	77 (±14)	6 (±5)	54 (±3)	11 (±1)	7[c]	3		
CB added Day 3			270 (±23)	55 (±15)	244 (±206)	107 (±78)		
CB added Day 4					708 (±205)	270 (±61)	583 (±30)	815 (±156)

[a] Effect of CB on Ig secretion by LPS-stimulated cells. Experiment I: 4×10^5 cells/ml were cultured. Five micrograms per milliliter of CB were added 12 hr before harvest each day. IgM and IgG_1 PFC were measured. Experiment II: 3×10^5 cells/ml were cultured. One microgram per milliliter of CB was added on Days 2, 3, or 4 and responses were measured at indicated days. IgM and total IgG PFC were measured.

[b] Arithmetical mean ± S.D. of triplicates.

[c] Three cultures were pooled before testing.

TABLE III

Development of IgM⁺, IgG⁺, or IgM⁺IgG⁺ containing cells in LPS cultures treated with CB[a]

CB Added?	Day of CB Addition	Day of Harvest	IgM⁺/Total Cells[b]	IgG^{+}_{2a}/Total Cells[b]	$IgM^{+}IgG^{+}_{2a}/IgG^{+}_{2a}$[c]	$IgM^{+}IgG_1^{+}/IgG_1^{+}$[c]
			×100	×100	×100	×100
No		2	6	<0.5	n.d.	n.d.
No		3	15	2	26 ± 4(300)	26 ± 4(100)
Yes	2	3			83 ± 4(900)	59 ± 6(150)
No		4	35	4	10 ± 1(400)	10 ± 3(150)
Yes	2	4			63 ± 10(500)	61 ± 6(150)
Yes	3	4			18 ± 3(400)	17 ± 5(200)
No		5	54	15	7 (200)	n.d.
Yes	4	5			27 (100)	n.d.
No		9[d]	28	27	<0.1 (1000)	0.5 (1000)

[a] One microgram per milliliter of CB was added at indicated times after onset of LPS-stimulated cultures. Cells were harvested 1 day (17 to 24 hr pulse) or 2 days (42 hr pulse) after CB treatment together with controls without CB addition. Cytocentrifuged smears were prepared as described in *Materials and Methods* and were sequentially stained with anti-IgG and anti-IgM.

[b] Cells were stained with TRITC-anti-IgM and FITC-anti-IgG_{2a}. The values are derived from one experiment. From 200 to 500 total cells were examined.

[c] Cells were stained with TRITC-anti IgM and FITC-anti-IgG_{2a} (left column) or with FITC-anti-IgM and TRITC-anti-IgG_1 (right column). The values represent the arithmetic mean ± S.E. of five consecutive experiments. The numbers in parentheses refer to the total numbers of IgG^{+} cells analyzed. Only cells with two or more nuclei were considered.

[d] Cells were subcultured at 4×10^5 cells/ml in fresh medium every 2nd day.

ever, when CB was added later, on Day 3 or Day 4, the percentage of double-staining cells found on Day 4 or 5, respectively, were low and approached control values although the frequency of polynucleated cells remained high.

This pattern of increased numbers of double staining, IgG-producing cells after early CB pulses was a reproducible finding in five consecutive experiments. A group of single and double staining cells is shown in Figure 3. Part of these double staining cells are dull for IgG and bright for IgM. In the data shown in Table III only bi- and multi-nucleated cells in CB-pulsed groups were counted. A similarly high fraction of doubles was found among the binucleated cells only. Among the mononucleated cells the percentage of doubles was not significantly different from that found in the controls.

DISCUSSION

As extensively documented (5, 9, 10–14) both IgM and IgG production can occur after LPS activation. The polyclonal plaque assay seems to be well suited for measuring Ig secretion in this system. Thus, an accurate determination of Ig-secreting can be obtained, even though the cells secreting the particular class of Ig is in minority. An example of this is shown in Figure 1, where an accurate measurement of the number of IgG-secreting cells could be obtained Day 2, although they were 1000-fold less frequent than the IgM-secreting cells at this time. The difference in kinetics between the IgM and the IgG response is interesting. IgM-secreting cells could both be detected earlier and also peaked earlier than IgG-secreting cells. Furthermore, whereas the IgM response increased approximately 40-fold during its period of exponential growth, the IgG response increased more than 1000-fold over a period of 2 days. This pattern strongly argues against the increased IgG response being merely the result of proliferating IgG-secreting cells, since this increase is too large to be accounted for by proliferation alone. In itself, the kinetic pattern can be taken as evidence that a switch from IgM to IgG occurs in this system.

After addition of CB to LPS-activated cells, the number of cells per culture failed to increase, and the numbers of cells decreased rapidly. It is interesting that a decrease and not a steady state in cell numbers were observed after CB. It is possible that this reflects toxicity of CB after prolonged exposure. Even though cell numbers did not increase after addition of CB, nuclear proliferation continued as determined by the extensive polynucleation. Janossy and Greaves (18) have shown that nuclei of LPS-activated, Ig secreting and nonsecreting cells divide at a similar rate using CB. By using the same technique, we found that IgM and IgG2a-containing cells had a similar degree of polynucleation. This provides direct evidence that the rapid increase in the proportion of IgG-producing cells, typical for this culture system, is not due to a preferential expansion of those cells.

Parkhouse and Allison (4) measured extracellular secretion of Ig by plasmacytomas and found no inhibition of secretion by CB (4). Measuring secretion of normal LPS-activated cells in the polyclonal plaque assay, we found no inhibition by short CB pulses. The reduced numbers of secreting cells after prolonged treatment were possibly related to toxicity of CB. That short CB pulses does not inhibit Ig secretion is noteworthy since it inhibits a number of other membrane-dependent processes, such as mobility, phagocytosis, and cytotoxic killing (1, 2, 19).

CB treatment led to a dramatic rise in the fraction of IgG-producing cells that also contained IgM. Since secretion seemed not to be inhibited, it is unlikely that the double staining cells only produced IgG, and merely retained previously synthesized IgM. Furthermore, the fraction of double staining cells remained high for a period of at least 42 hr. Thus, the double staining cells were most likely synthesizing both isotypes simultaneously. There is no evidence that CB influences transcriptional or translational events, and it seems likely that each nucleus in the multinucleated cells behaves as in untreated cells and synthesizes messenger for only one isotype of secretory Ig.

The evidence is increasing that a switch from IgM to IgG occurs *in vitro,* after LPS stimulation in the low cell density cultures. Thus, Pernis *et al.* (10) and Kearny *et al.* (11) demonstrated the existence of cells containing both IgM and IgG, occurring after LPS stimulation of mouse spleen cells. Using single cell techniques, Wabl *et al.* (12) and Anderson *et al.* (13) recently have shown that the lineage of one cell can synthesize and secrete both IgM and IgG. The present results are in complete agreement with the latter findings. Furthermore, our results might give some indication as to how the switch occurs. There is an interesting relation between the kinetics of the IgG response and the effect of CB addition at different times. Thus, we only observed an increase in the fraction of double producers when CB was present over the time when the first IgG-producing cells appear, i.e., between Days 2 and 3. We propose that cells in the process of switching have to go through an asymmetric division leading to one daughter cell producing the new isotype (IgG), and the other producing IgM. This would explain the results of CB treatment at early times. Furthermore, we suggest that each cell type would be self-renewing. The IgG-producing, proliferating cells would soon outnumber the cells that switch at later times. Thus, CB treatment at those times would still lead to double producers among the switching cells, but since the latter would be in the minority, no dramatic

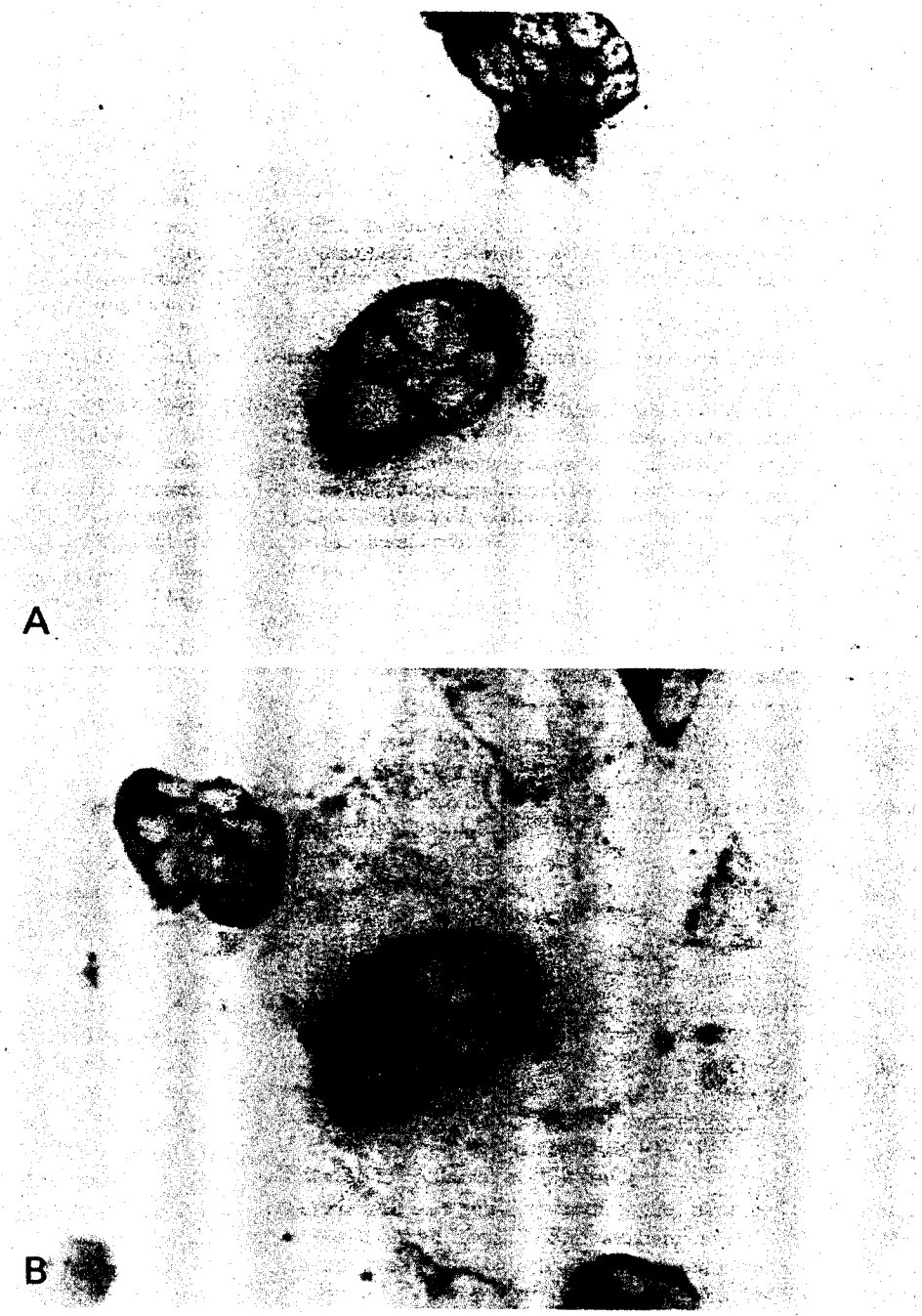

Figure 3. Immunofluorescent staining for cytoplasmic immunoglobulin of LPS activated cells treated with CB. Addition of CB blocks cytoplasmic cleavage of dividing cells so that multinucleated cells are formed (Table I). In LPS-stimulated cultures, addition of CB led to an increased ratio of cells with both IgM and IgG in their cytoplasm (Table II). The photomicrograph shown was obtained from cultures harvested at Day 4 at the end of a 40-hr pulse with 1 μg/ml of CB. Cells were stained with fluorochrome-labeled class-specific antisera as described in Table II. Figures 1*A* and 1*B* represent negative prints of the same field photographed under selective filtration. *A*, field under red filtration revealing the cytoplasm of cells staining with rhodamine-conjugated goat-anti-mouse-IgG; *B*, field under green filtration revealing the cytoplasm of cells staining with fluorescein-conjugated goat-anti-mouse-IgM. The cell in the center is stained with both reagents (Original magnification × 2000).

increase in the double producing cells would be observed.

It is more difficult to postulate why only one of two daughter cells would express the new phenotype. It remains unclear whether the switch from IgM to IgG involves genetic translocation, or merely activation of a preexisting genotype (20). The idea that the switch involves an asymmetric division does not allow a distinction between these possibilities. However, it agrees well with a mechanism of semi-conservative replication. Furthermore, asymmetric division has been shown to occur in other differentiation systems [for discussion of these topics, see (21)]. This report illustrates how CB can be a useful tool for studying cell differentiation processes in the immune system. We suggest it will have similar uses in other differentiating systems.

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