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Mediators from cloned T helper cell lines affect immunoglobulin expression by B cells

(B cell lines/T cell supernatants/immunoglobulin synthesis and secretion)

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ABSTRACT **When cloned T helper cells encounter antigen presented by I-A-compatible macrophages, soluble mediators are produced that affect the differentiation and activation of normal B lymphocytes and cell lines of the B lineage. Exposure to such T cell culture supernatants causes two effects in the murine 70Z/3 cell line, which represents a pre-B stage of differentiation. These cells begin to synthesize Ig light chains and gain membrane Ig that is detectable by immunofluorescence. Two other effects are seen after similar treatment of the WEHI-279.1 murine cell line, which represents a mature, Ig^+ B cell. These cells shift the ratio of μ chains produced from mostly membrane to mostly secretory type and begin to secrete large amounts of IgM, which can be detected either by biosynthetic radiolabeling followed by immunoprecipitation or by a staphylococcal protein A plaque assay. The majority also die. Similar to WEHI-279.1, normal small resting B cells also show the shift from membrane μ to secretory μ and are activated to Ig secretion after exposure to these supernatants. These results show that products from T cell immune reactions exert multiple effects on B cell development and activation, at several stages of the B cell developmental pathway. The observed changes range from nuclear processes, including gene transcription and RNA splicing, to such post-translational aspects as protein processing, catabolism, membrane architecture, and cell survival.**

Supernatants of stimulated lymphoid cell cultures contain biologically active factors that influence the growth and differentiation of B lymphocytes (reviewed in refs. 1 and 2). In the past, it has been difficult to precisely define the biological function of these activities and determine at which level of B cell development they operate. In part this is due to the complex cellular systems that have been used (3). Some of these difficulties have been overcome by the use of supernatants of cloned, antigen-specific T cell lines, cultured in the presence of appropriate antigen and histocompatible accessory cells (4, 5).

The first recognizable cells of the B lineage are cytoplasmic Ig^+ cells that synthesize μ heavy (H) but not light (L) chains and are found in fetal liver (6, 7). The asynchronous onset in Ig chain synthesis is reflected at the level of DNA, because the genetic rearrangements necessary for Ig expression occur first for the μ gene and subsequently for the L chain gene (8, 9). It is believed that these "μ-only" cells give rise to progeny with fully rearranged L chain genes, although L chain may not yet be synthesized (8, 10). At this stage μ chains of the membrane form may in fact be present on the cell surface in the absence of L chain (10, 11). The following stage of differentiation is heralded by the onset of L chain synthesis and the expression of complete IgM molecules on the cell membrane. These surface-Ig^+ (sIg^+) cells are small and quiescent, in contrast to earlier stages, which are composed of larger proliferating cell types (12). At this point in B cell development, the entire membrane μ biosynthetic pathway is operational, while only the early stages of the secretory μ pathway are present (13). Subsequent to activation, additional post-translational processes generate the missing secretory μ forms and allow IgM secretion (13). Finally, in the most mature cells of the B lineage, Ig-secreting plasma cells, the membrane μ pathway may be reduced or deleted (13–15).

In the present report, we demonstrate that factors contained in the supernatants of antigen-stimulated cloned T cells cause differentiation of established cell lines of the B lineage. The pre-B cell line 70Z/3 (10, 16), which under normal growth conditions synthesizes immunoglobulin μ H chains but not L chains, is induced to synthesize L chains, which are subsequently expressed on the cell surface. The B cell lymphoma WEHI-279.1 (17, 18), which under normal growth conditions synthesizes and displays membrane IgM while secreting only low amounts of IgM extracellularly, is induced to secrete large amounts of IgM. Studies with both small resting splenic B cells and WEHI-279.1 also demonstrate a rise in the secretory-to-membrane μ ratio and increased cell death after exposure to T cell supernatants. These assays provide easy and reproducible means to screen small amounts of biologically active material and also allow the detailed analysis of the effects of soluble factors on multiple stages of B cell differentiation.

MATERIAL AND METHODS

B Cell Lines. The murine pre-B cell line 70Z/3 was established *in vitro* from the spleen of a thymectomized (C57BL/6 × DBA/2)F_1 mouse previously exposed to methylnitrosourea (16, 19). The growth properties of this line and its response to mitogen have been described (10, 16). Murine WEHI-279.1 cells were a generous gift from W. Raschke (La Jolla, CA) and have also been previously described (17, 18). Additional murine B cell lines tested in these experiments include WEHI-231 (20), GCL-2.8 (21), and 38C-13 (22). These lines were routinely cultured in Iscove's modified Dulbecco's medium (IMD medium) supplemented with human transferrin at 5 μg/ml, 0.5% delipidated bovine serum albumin, and 5% fetal calf serum (Boehringer Mannheim no. 66459502).

T Helper Cell Lines. Helper T cell lines were established by repeated antigen stimulation *in vitro* in the presence of irradiated spleen cells, cloned under conditions of limiting dilution, and propagated in T cell growth factor (TCGF)-supplemented medium as described (23, 24). The supernatants used in this study were derived from 60-hr serum-free cultures con-

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Abbreviations: L and H chains, light and heavy chains of immunoglobulins; μ_s and μ_m, secretory and membrane μ H chains; sIg, surface immunoglobulin; IMD medium, Iscove's modified Dulbecco's medium; SRBC, sheep erythrocytes; OVA, ovalbumin; PE, peritoneal exudate cells; LPS, lipopolysaccharide; PFC, plaque-forming cells; kDal, kilodalton(s).

taining (per ml) 2.5×10^4 sheep erythrocyte (SRBC)-specific helper T cells (C57BL/6) or 5×10^4 chicken ovalbumin (OVA)-specific helper T cells (C57BL/6), the antigen SRBC (2.5×10^6) or OVA (100 μg), and 1×10^4 C57BL/6 *nu/nu* spleen cells or 5×10^4 peritoneal exudate cells (PE) derived from either C57BL/6 normal (+/+) or nude (*nu/nu*) mice or the indicated congeneic mouse strains.

Cells and Cultures. In most experiments 1–5 $\times 10^5$ WEHI-279.1 cells or 5×10^4 70Z/3 cells were grown in 0.5 ml of medium in 24-well tissue culture plates (Costar, Cambridge, MA). Small spleen cells were isolated from erythrocyte-depleted populations at ≥ 1.08 g/cm^3 in Percoll density gradients and cultured at a concentration of 10^6 per ml. To each well was added either T cell supernatant or a similar volume of medium. The cells were incubated for 48 hr at 37°C in 5% CO_2. After culture the cells were harvested and washed once in Earle's balanced salts solution, and viable cells were counted on the basis of trypan blue exclusion. Aliquots were used for the various assays. Similar cultures were exposed to lipopolysaccharide (LPS, from *Salmonella typhosa* WO901, Difco) at 10–100 μg/ml for cell lines and 50 μg/ml for small spleen cells. 70Z/3 cells and small spleen cells consistently responded to LPS under these conditions. The response of WEHI-279.1 cells to LPS was more variable and was found to be dependent on undefined factors present in different batches of fetal calf serum (unpublished results). All batches of fetal calf serum tested were able to support LPS responses of spleen cells and 70Z/3 cells. Small spleen cells, 70Z/3 cells, and WEHI-279.1 cells consistently responded to T cell supernatants in all batches of fetal calf serum tested and serum-free conditions.

Immunofluorescence. The sIg expression of 70Z/3 cells was determined by using a rhodamine-conjugated anti-μ reagent prepared from a rabbit anti-MOPC 104E antiserum and enriched by binding to staphylococcal protein-A-conjugated Sepharose (donated by L. Forni, Basel). Generally, 5×10^5 cells were washed once and resuspended in 50 μl of staining reagent for 15 min on ice. They were subsequently washed once with IMD medium, once through 100% fetal calf serum, and a third time with IMD medium containing 2% fetal calf serum. The cells were then resuspended in one or two drops of IMD medium containing 2% fetal calf serum and examined with a Zeiss Photomicroscope.

Plaque Formation. Protein A-coupled SRBC were prepared according to the method of Gronowicz *et al.* (25). The reverse plaque assay, using appropriately diluted rabbit anti-mouse IgM antiserum (anti-MOPC 104E) and selected guinea pig complement (GIBCO), was used as described (25).

Biochemical Analysis. After culture, cells were washed twice in Earle's balanced salts solution. Equal numbers of cells were labeled with [^{3}H]leucine or [^{35}S]methionine, in medium deficient in that amino acid, for 1 hr in the absence of tunicamycin (for the patterns of glycosylated molecules in cell lysates), for 4 hr in the absence of tunicamycin (for secreted glycosylated molecules in culture supernatants), or for 3 hr in the presence of tunicamycin at 5 μg/ml (after 1-hr preexposure to tunicamycin) (for the pattern of nonglycosylated molecules in cell lysates). After these labeling periods, culture supernatants or nonionic detergent cell lysates were immunoprecipitated with rabbit anti-mouse IgM (μ and κ) antiserum and protein A-Sepharose CL-4B beads, eluted, electrophoresed on 20-cm NaDodSO$_4$/linear 8–18% polyacrylamide gradient gels, and fluorographed. Some gel bands were excised and their radioactivities were measured. These procedures may be found in detail in ref. 13.

RESULTS

Exposure of the pre-B cell line 70Z/3 to supernatants derived from T helper cells stimulated by antigen in the presence of I-A-compatible accessory cells resulted in a significant increase in sIg expression as judged by immunofluorescence. Data obtained with two helper T cell clones (24), OVA-7, specific for OVA, and S26-5, specific for SRBC, are shown in Table 1. These data indicate that the induction of active T cell supernatants was antigen specific and required I-A-compatible accessory cells.

We also investigated the differentiation of B lymphocytes in response to T cell supernatants by biosynthetically labeling B cells with radioactive amino acids and examining the resulting intracellular and secreted molecules by immunoprecipitation and NaDodSO$_4$/polyacrylamide gel electrophoresis. Results obtained with small splenic B cells and with cell lines 70Z/3 and WEHI-279.1 are shown in Fig. 1.

The first radiolabeling protocol was a 1-hr incubation in medium containing radioactive amino acids followed by examination of the intracellular Ig molecules. Previous studies have shown that no detectable processing or secretion of radiolabeled Ig molecules occurs in this period and that the resulting patterns, especially of μ chains, are diagnostic of the B cells' state of differentiation (13). As expected, small (resting) splenic B cells produced one major glycosylated intracellular μ species, of apparent molecular mass 73 kilodaltons (kDal) (Fig. 1, 1-hr lysate). After exposure to LPS or T cell supernatants, the two glycosylated intracellular μ species (73 and 70 kDal) characteristic of activated B cells were seen instead. It has been previously demonstrated that the 70-kDal form is unique to the secretory pathway, whereas both secretory and membrane μ have 73-kDal forms (13). T cell supernatants, but not LPS, also increased the 70-kDal form in WEHI-279.1 cells. Neither T cell supernatant nor LPS detectably altered μ chain biosynthesis in 70Z/3 cells, although both induced the synthesis of L chains (25 kDal). (The induction of L chains in 70Z/3 is better seen in the 3-hr radiolabeling results with tunicamycin—see below.) The induction of the activity that causes L chain synthesis also required antigen-mediated, I-A-restricted T cell–macrophage interaction (Table 1).

Table 1. Effects of T cell supernatants on sIg and L chain expression in 70Z/3 cells

T cells	Antigen	Accessory cells	% sIg$^+$	Presence of L chain
OVA-7	OVA	B6 +/+ PE	81	+
OVA-7	OVA	B6 *nu/nu* PE	88	+
OVA-7	OVA	None	12	−
OVA-7	OVA	B10.A (4R) PE*	21	−
OVA-7	OVA	B10.A (5R) PE	83	+
OVA-7	OVA	B10.MBR PE*	13	−
OVA-7	None	B6 *nu/nu* PE	18	−
OVA-7	SRBC	B6 *nu/nu* spleen	16	−
S26-5	SRBC	B6 *nu/nu* spleen	82	+

70Z/3 cells (5×10^4 per ml) were cultured for 48 hr in IMD medium containing 10% fetal calf serum and 20% supernatants derived from 60-hr cultures containing, per ml, 2.5×10^4 SRBC-specific helper T cells (S26-5) or 5×10^4 OVA-specific helper T cells (OVA-7), the antigen [SRBC (2.5×10^6) or OVA (100 μg)], and 5×10^4 PE of the indicated congeneic mouse strains or 1×10^4 C57BL/6 (B6) *nu/nu* spleen cells. sIg expression was determined by using rhodamine-conjugated rabbit anti-mouse μ antibody. The presence of L chain was determined by following the protocol described for Fig. 1. Uninduced control cultures were 10% sIg$^+$ and L chain negative.

* These strains are *I*-region nonidentical with the C57BL/6 helper T cell clones.

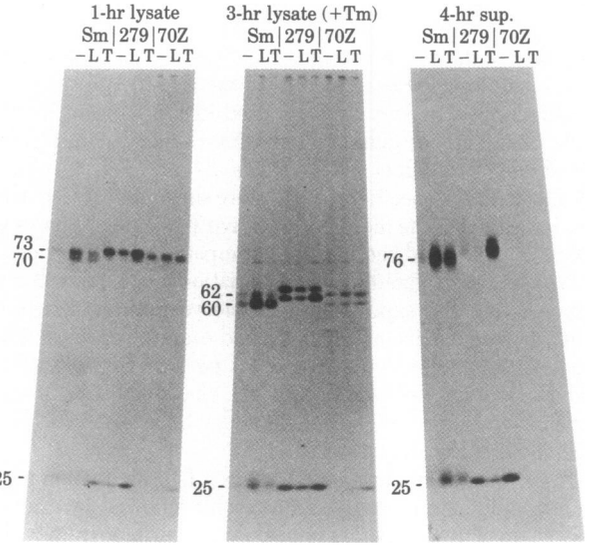

FIG. 1. Biochemical analysis of Ig production. Small spleen cells (Sm), WEHI-279.1 (279), and 70Z/3 (70Z) were cultured for 48 hr in the presence of LPS (L), 20% T cell supernatant (T), or without added stimulus (−). The first panel (1-hr lysate) shows the electrophoretic patterns of the intracellular glycosylated molecules. The second panel [3-hr lysate (+Tm)] shows the intracellular nonglycosylated molecules labeled for 3 hr in the presence of tunicamycin (5 μg/ml) (after 1 hr preexposure to tunicamycin). The third panel (4-hr sup.) shows the pattern of secreted glycosylated molecules in the culture supernatants. Molecular masses in kDal are indicated.

Because the synthesis of the intracellular 70-kDal μ form correlates with and is probably necessary for IgM secretion (13), we examined culture supernatants for secreted IgM (Fig. 1, 4-hr sup.). Both LPS and T cell-derived supernatants greatly increased the IgM secretion of small splenic B cells. (In the experiment shown, 2,900, 28,100, and 17,000 cpm of μ chain were secreted by control, LPS- and T cell supernatant-treated cells, respectively.) Cell line WEHI-279.1 responded with increased μ secretion to T cell supernatants but not to LPS (2,100, 1,100, and 30,900 cpm of μ chain, respectively). Cell line 70Z/3 never showed appreciable μ secretion (600, 400, and 300 cpm of μ, respectively).

We also measured the relative amounts of membrane and secretory μ (μ_m and μ_s, respectively) produced by examining the nonglycosylated polypeptides made in the presence of tunicamycin. Both forms (62 and 60 kDal) are present in resting B cells, but the ratio changes in favor of the secretory form (60 kDal) after B cell activation (13), probably reflecting changes in the corresponding mRNA populations (14, 15). The ratio of secretory to membrane μ made in small B cells changed in response to both LPS and T cell supernatants [1.4, 3.0, and 3.0 μ_s per μ_m in small B cells treated with medium alone, LPS, and T cell supernatants, respectively, in the experiment shown in Fig. 1, 3-hr lysate (+Tm)]. In WEHI-279.1 populations, the secretory-to-membrane μ ratio changed only in response to T-cell supernatants and not to LPS (0.67, 0.72, and 2.9 μ_s per μ_m, respectively). With 70Z/3 populations, neither treatment changed the secretory-to-membrane μ ratio (0.45, 0.41, and 0.52 μ_s per μ_m, respectively).

The effect of T cell supernatants on WEHI-279.1 cells was also demonstrated in a protein A reverse plaque assay (Table 2). Both plaque formation and the shift in the proportions of μ forms required T cell supernatants generated with I-A-compatible accessory cells and appropriate antigen. We also observed that the recovery of WEHI-279.1 cells was greatly diminished in the cultures containing active T cell supernatants, as compared to noninduced control cultures. This effect is due to extensive cell death rather than lack of proliferation, because a large number of nonviable cells were detected in these cultures.

Table 2. Effects of T cell supernatants on the growth and Ig expression of WEHI-279.1 cells

T cells	Antigen	Accessory cells	PFC/10^6 viable cells	μ_s/μ_m	Cells recovered $\times 10^{-6}$
OVA-7	OVA	B6 +/+ PE	900	2.2	1.1
OVA-7	OVA	B6 *nu/nu* PE	1,100	2.8	1.2
OVA-7	OVA	None	3	0.56	13
OVA-7	OVA	B10.A (4R) PE*	150	1.0	4.6
OVA-7	OVA	B10.A (5R) PE	1,200	4.0	2.3
OVA-7	OVA	B10.MBR PE*	100	1.5	8.1
OVA-7	None	B6 *nu/nu* PE	8	0.75	11
OVA-7	SRBC	B6 *nu/nu* spleen	30	0.96	9.5
S26-5	SRBC	B6 *nu/nu* spleen	2,200	2.4	1.6

WEHI-279.1 cells (5×10^4 per ml; 6.25×10^5 total cells) were cultured for 48 hr in IMD medium containing 10% fetal calf serum and 25% supernatants, as described for Table 1. Plaque-forming cells (PFC) were determined in a reverse staphylococcal protein A assay (25). The ratio of μ_s to μ_m was determined. Uninduced control WEHI-279.1 cells generated 6 PFC/10^6 viable cells and $\mu_s/\mu_m = 0.96$.
* These strains are *I*-region nonidentical with the C57BL/6 (B6) helper T cell clones.

Fig. 2 details several aspects of the response of WEHI-279.1 cells to S26-5 supernatants. Fig. 2*C* shows that 1% supernatant induced significant plaque formation per culture, and that plateau values were reached by 7.5% supernatant. The minimal cell recovery, shown in Fig. 2*B*, occurred at 15% supernatant.

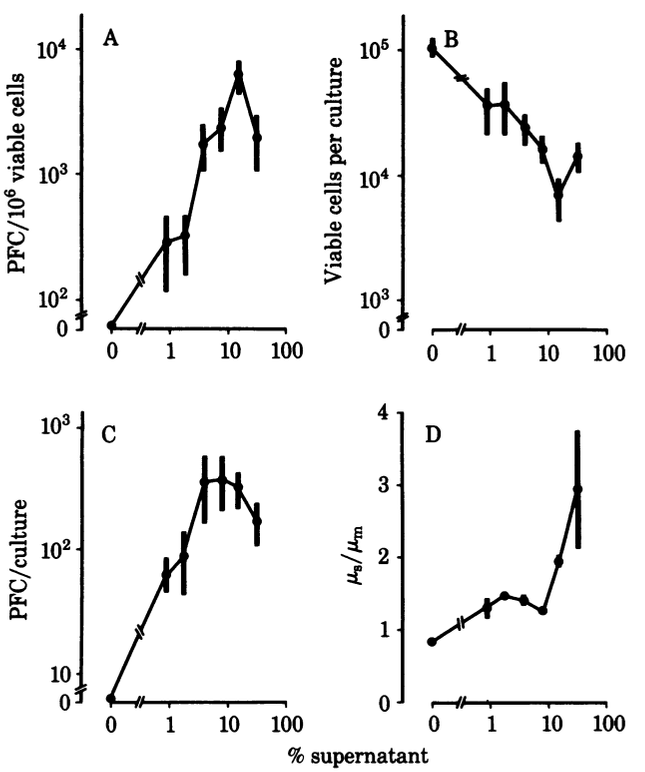

FIG. 2. Response of WEHI-279.1 cells incubated for 48 hr with increasing amounts of S26-5 T cell supernatant. Mean values ± SEM are presented. (*A*) Number of PFC/10^6 viable cells. (*B*) Total number of viable cells recovered per culture. (*C*) Total number of PFC/culture. (*D*) μ_s/μ_m. Total μ cpm were 3,200 ± 270 for 0% supernatant and 190 ± 10 for 9% supernatant.

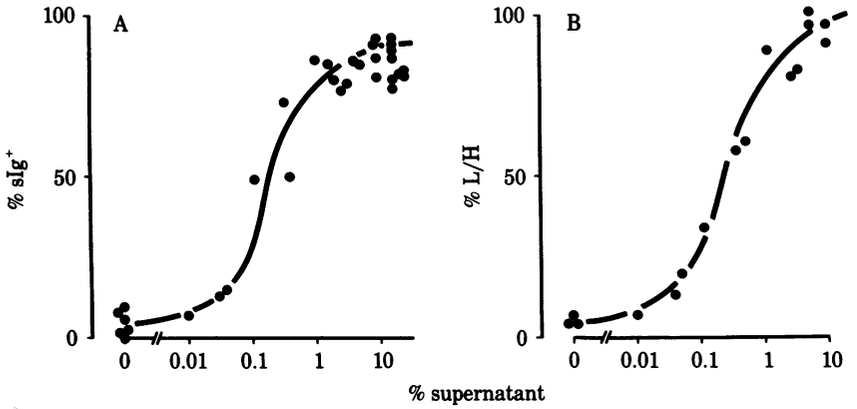

FIG. 3. Response of 70Z/3 cells incubated for 48 hr with increasing amounts of S26-5 supernatant. Data points are individual experimental values. (*A*) Percent sIg^+ cells. (*B*) Amount of L chain synthesis relative to total μ chain synthesis. Total μ synthesis did not appreciably change in induced vs. noninduced cells: noninduced, 2,500 ± 290 cpm; 9% supernatant, 1,700 ± 180 cpm per culture. The comparable L chain values were 190 ± 60 for noninduced and 1,600 ± 180 for 9% supernatant.

When these data are combined to yield the number of plaques per 10^6 viable cells (Fig. 2*A*), maximal levels were reached at 15%, reflecting the cell recovery pattern. The increase in the μ_s/μ_m ratio is shown in Fig. 2*D*; it continued to rise with increasing amounts of supernatant.

The response of 70Z/3 cells to increasing concentrations of S26-5 supernatants is depicted in Fig. 3. This shows that 1% supernatant was sufficient to reach plateau levels for both sIg expression (Fig. 3*A*) and L chain synthesis (Fig. 3*B*), and that the activity in even supernatant diluted 1:1,000 (0.1%) was readily detectable.

We also analyzed the induction of 70Z/3 and WEHI-279.1 cells over a 4-day time course (Fig. 4). The percentage of sIg^+ 70Z/3 cells reached maximal levels by 24 hr (Fig. 4*A*) while L chain synthesis (Fig. 4*B*), although substantial after 24 hr, required 48 hr to reach maximum. Similarly, the change in the WEHI-279.1 μ_s/μ_m ratio was virtually complete by 24 hr (Fig. 4*D*), but the PFC response required 48 hr to develop fully (Fig. 4*C*).

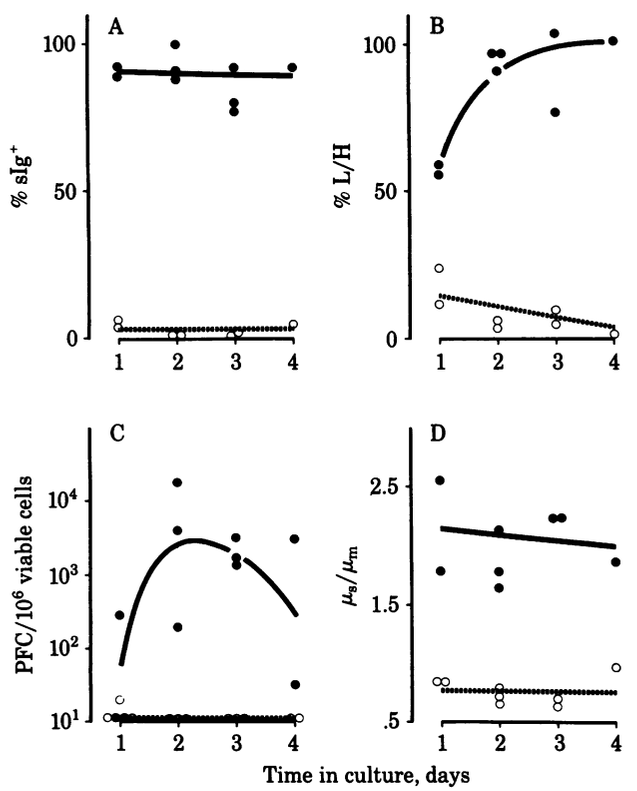

FIG. 4. Kinetics of responses to S26-5 supernatant. The supernatant concentrations used with WEHI-279.1 and 70Z/3 cells were 15% and 9%, respectively. Data points represent individual experiment values. (*A*) Percent sIg^+ 70Z/3 cells. (*B*) Amount of L chain synthesis by 70Z/3 cells in relation to total μ synthesis. During this time period μ synthesis by 70Z/3 cell cultures remained constant within a factor of 2. Average μ chain radioactivity per induced culture on days 1–4 was 2,060, 1,700, 1,200, and 880 cpm, respectively, and the comparable L chain values were 1,200, 1,600, 1,400, and 1,000 cpm. Noninduced cultures produced 250 cpm or less of L chain. (*C*) Plaque formation by WEHI-279.1 cells, expressed as PFC/10^6 viable cells recovered. (*D*) μ_s/μ_m in WEHI-279.1 cells.

DISCUSSION

Previous studies have demonstrated that various biologically active factors are found in supernatants of appropriately stimulated helper T cells. These activities can be measured in several *in vitro* systems including: (*i*) propagation of cloned T cell lines (26, 27); (*ii*) replacement of T cells in antibody responses to certain antigens (23, 24, 27); (*iii*) maintenance of antigen-independent proliferation of B cell blasts (4); (*iv*) maturation of small resting B cells to antibody secretion without proliferation (5); and (*v*) stimulation of granulocyte, macrophage, and erythrocyte progenitors to proliferate in semisolid medium (27–29).

The observations reported here show that these supernatants also contain molecules that affect multiple stages of Ig expression by B cells and B cell lines. In the pre-B cell line 70Z/3, T cell supernatants, like LPS, cause L chain synthesis and sIg expression that is detectable by immunofluorescence. The first effect is most likely due to a transcriptional change of the L chain genes, because noninduced 70Z/3 cells contain very little L chain mRNA (30, 31). The absolute amount of L chain synthesized per 70Z/3 culture rose at least 10–20 times in these experiments, while the amount of μ produced stayed constant within a factor of 2. The second change, the development of sIg detectable by immunofluorescence, may merely represent an altered orientation of preexisting cell surface μ chains, because these are detectable on noninduced 70Z/3 cells by surface iodination and immunoprecipitation but not by immunofluorescence (unpublished data). However, if these two changes are directly related, it may be of interest that the maximal number of sIg^+ cells was reached prior to maximal L chain synthesis. One should point out that the percentage of cells detected as Ig^+ is not a quantitative measure of the amount of surface IgM per cell. We do not yet know when maximal IgM levels per cell are reached.

At the following stage of B cell differentiation, that of the resting, sIg^+ B lymphocyte, T cell supernatants yield two other effects on Ig expression. One is a change in the proportion of membrane μ synthesis to secretory μ synthesis in favor of secretory μ. This may be mediated by a perturbation in the RNA splicing mechanism that produces the alternative mRNA spe-

cies (14, 15). It should be noted that the total amount of μ synthesized per viable WEHI-279.1 cell did not increase significantly during this transition. The final effect on nonsecreting B cells is the completion of secretory μ post-translational processing, as evidenced by the 70-kDal intracellular μ species and active IgM secretion (13). Maximal PFC per culture were reached with only a modest μ_s/μ_m ratio change in the titration experiment, while maximal μ_s/μ_m enhancement occurred before significant PFC induction in the kinetic experiment. These observations suggest that these two changes are distinct.

The cell death caused by S26-5 supernatants is not yet understood. All tested T cell supernatants caused this effect, and it was seen over a wide range of initial cells per culture (5×10^4 to 2×10^6). The high number of dead cells counted in the cultures indicates that proliferation did occur (usually 3- to 4-fold in 48 hr). The possibility that the induction of plaque formation could be accounted for by selection and enrichment of the rare PFC in the uninduced population is ruled out by the very low number of background plaques ($<10/10^6$) when cultures were initiated, and the absolute increase in PFC/culture (Fig. 2*C*). These data may reflect a normal aspect of *in vivo* differentiation to Ig secretion and have therapeutic application as well. In our hands, other B cell lines tested [WEHI-231 (20), GCL-2.8 (21), and 38C-13 (22)] were unresponsive, in all assays, to both LPS and T cell supernatants.

Other reports have shown changes in Ig expression by B cell lines stimulated by polyclonal mitogens (16, 31–39) or supernatants from complex mitogen-induced cell cultures (40–42), but factors derived from antigen-induced helper T cell cultures or monoclonal T cell lines in general may be more physiologically relevant and definable. At present, we do not know whether the many effects demonstrated here result from one or several separate biologically active factors, or whether all of the observed changes within a given responding B cell population are aspects of a single integrated response pathway. These readily measurable changes in stable B cell lines should, however, provide sensitive assays for antigen-induced soluble factors and the proper tools for biochemical studies resolving their nature and mechanism of action.

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