# CLONAL ANALYSIS OF B CELLS INDUCED TO SECRETE IgG BY T CELL-DERIVED LYMPHOKINE(S)

BY JUDITH E. LAYTON,* ELLEN S. VITETTA,* JONATHAN W. UHR,* AND PETER H. KRAMMER‡

*From the *Department of Microbiology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235; and the ‡Institute for Immunology and Genetics of the German Cancer Research Center, Heidelberg, Federal Republic of Germany*

The switch from IgM to IgG (or IgA, IgE) synthesis has been studied in both normal and neoplastic Ig-secreting cells (1–6). Evidence suggests that DNA rearrangements precede isotype switching. The nature of the signals that induce isotype switching are not understood, although T cells (7–13), T cell–derived lymphokines (14–20), and mitogens (20–22) can influence switching in activated B cells.

We have previously (16) described a T cell–derived lymphokine termed "B cell differentiation factor for IgG" (BCDF$\gamma$),[1] that induces increased levels of IgG1 secretion in lipopolysaccharide (LPS)-stimulated splenic B cells. T cell supernatants (SN) containing BCDF$\gamma$ also contain lymphokines that suppress the secretion of IgG3 (23) and IgG2 (24, 25). These changes in IgG secretion are accompanied by corresponding changes in the steady state levels of mRNA for each IgG subclass (23). BCDF$\gamma$-containing SN do not act on cells that bear surface IgG (sIgG) at the initiation of culture (16). Furthermore, BCDF$\gamma$ does not bind to Sepharose-coupled IgM, IgD, or IgG (25). The cell surface receptor for BCDF$\gamma$, then, is probably not sIg.

The mechanism of action of BCDF$\gamma$ is unclear. It might induce growth of an sIgG$^+$ subset of B cells. Alternatively, BCDF$\gamma$ might induce sIgG$^-$ B cells to secrete IgG1. In the present studies, we have examined the effects of a BCDF$\gamma$-containing T cell SN on normal B cells in a limiting dilution culture system. Our results suggest that BCDF$\gamma$-containing SN induce IgG1 secretion in a subset of sIgG$^-$ B cells already committed to a differentiation pathway leading to IgG1 secretion. These cells do not arise from precursors of IgG3-secreting cells.

## Materials and Methods

*Animals.* We used female C57BL/6 × DBA/2 $F_1$ ($BDF_1$) mice (The Jackson Laboratory, Bar Harbor, ME) 8–16 wk old. Lewis rats (Harlan Sprague Dawley, Inc., Indianapolis, IN), used at 3–6 wk, were a source of thymus cells. Rats were given tetracycline in their

This work was supported by grant AI-11851 from the National Institutes of Health and NATO grant No. 313-84.

[1] *Abbreviations used in this paper:* BCDF, T cell–derived B cell differentiation factor; BCGF, B cell growth factor, Con A, concanavalin A; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; GARIg, goat anti-rabbit Ig; IL, interleukin; LPS, lipopolysaccharide; PFC, plaque-forming cells; RAM$\gamma$, rabbit anti-mouse $\gamma$ chain; RIA, radioimmunoassay; sIg, surface Ig; SN, supernatant.

 J. EXP. MED. © The Rockefeller University Press · 0022-1007/84/12/1850/14 $1.00
Volume 160 December 1984 1850–1863

drinking water (500 mg/pint) for the first 2 wk after arrival to prevent mycoplasma infections.

*Cell Preparation.* T cell–depleted spleen cells (B cells) were prepared by treating cell suspensions with anti-Thy-1.2 monoclonal antibody HO-13.4 and baby rabbit complement (Pel-Freez Biologicals, Rogers, AR). This treatment abolished the proliferative response to concanavalin A (Con A). Rats were anesthetized with ether and exsanguinated from the axillary artery before removal of the thymus. Thymus cell suspensions were used as filler cells in the limiting dilution cultures.

*Specificity of the Anti-γ Antisera Used for Fluorescence.* $F(ab')_2$ fragments of rabbit anti-γ antibodies were prepared as described previously (26). Lymphoma cell lines were used for fluorescence analysis of these anti-γ antibodies. An IgG2a-bearing subclone of A20 (27) was obtained from Dr. Charlotte Word, UTHSC, Dallas. Two subclones, $BCL_{2a}$ ($sIgG1^+$, $sIgM^+$) and $BCL_{2b}$ ($sIgM^+$), derived from the in vitro $BCL_1$ cell line (28), were provided by Dr. Yung-Wu Chen, UTHSC, Dallas (manuscript in preparation). An in vitro adapted clone of the AKR/J B lymphoma 225 was developed by Dr. K. Brooks, UTHSC, Dallas, from the in vivo lymphoma obtained from Dr. E. Ritchie, M. D. Anderson Hospital, Houston. This clone expresses sIgM and sIgD (K. Brooks, personal communication).

Two control experiments were performed to establish the reactivity of the anti-γ sera with sIgG: (*a*) Two cell lines bearing sIgG (A20 and $BCL_{2a}$), normal splenic B cells stimulated with LPS for 5 d, and control $sIgG^-$ cell lines ($BCL_{2b}$ and AKR/J-225) were stained with $F(ab')_2$ rabbit anti-mouse γ chain (RAMγ) and fluorescein isothiocyanate–goat anti-rabbit Ig (FITC-GARIg). Stained cells were analyzed on an Ortho 50H cytofluorograph (Ortho Diagnostic Systems, Inc., Westwood, MA). The $sIgG^+$ cell lines were strongly positive, and the day-5 LPS blasts contained ~20% $sIgG^+$ cells. The intensity of staining of the LPS blasts was similar to that of the $sIgG^+$ cell lines, and <5% of the $sIgG^-$ cells and cells stained with FITC-GARIg alone were positive. (*b*) 6-d LPS blasts were stained, sorted into $sIgG^+$ and $sIgG^-$ populations, and analyzed for IgG3 plaque-forming cells (PFC). The staining had no effect on the total number of PFC obtained (25 PFC/$10^3$ cells). The sorted $sIgG^+$ cells gave a 5.5-fold higher PFC response than the $sIgG^-$ cells. Thus, staining with our antisera effectively identified and separated the $sIgG^+$ cells in the sorting procedure.

*Culture Conditions.* For limiting dilution cultures, varying numbers of splenic B cells were cultured with $3 \times 10^6$ thymus cells/ml in RPMI 1640 medium with 20 mM Hepes, penicillin (50 u/ml), streptomycin (50 μg/ml), gentamycin (10 μg/ml), L-glutamine (2 mM) (Gibco Laboratories, Grand Island, NY), 50 μM 2-mercaptoethanol, and 10% fetal calf serum (Hyclone; Sterile Systems, Inc. Logan, UT). 96-well, flat-bottomed microtiter plates (Costar, Cambridge, MA) were used. 48 wells, containing 0.2 ml cultures, were used for each concentration of B cells plated. A group containing no splenic B cells was included in each experiment to determine the background response from the thymus filler cells. This background was subtracted from the response of groups containing splenic B cells. Ig secretion was usually not observed. Cultures were incubated at 37°C in a humidified atmosphere of 83% $N_2$, 7% $O_2$, and 10% $CO_2$ for 6 d. B cells were stimulated with 20 μg/ml LPS (*Salmonella typhosa*; Difco Laboratories Inc., Detroit, MI) with or without a source of BCDFγ. BCDFγ-containing SN was added after 1 d of culture. In some experiments, B cells were cultured at a concentration of $2.5 \times 10^5$ cells/ml with 20 μg/ml LPS for 2–3 d. This was done in 75-$cm^2$ tissue culture flasks (Corning Glass Works, Corning, NY) to generate blasts for cell sorting.

*T Cell SN.* Cells from the cloned alloreactive AKR anti-C57BL/6 T cell line, PK 7.1, were maintained in culture as previously described (29). We induced lymphokine secretion by pulsing cells with 10 μg/ml Con A for 4 h, washing, and reculturing cells for 24 h in Con A–free medium. Such SN contain B cell growth factor (BCGF), B cell differentiation factor for IgM (BCDFμ) and IgG (BCDFγ), colony-stimulating factors, and histamine-producing-cell–stimulating factor (HCSF or interleukin 3 [IL-3]), but not γ-interferon, or conventional (30) T cell–replacing factor (TRF) (16, 29, 31).

*PFC Assay.* We performed reverse PFC assays in 96-well microtiter plates (Linbro Chemical Co., Hamden, CT) by the method of Pike et al. (32). We prepared protein A–

coupled sheep erythrocytes by the method of Gronowicz et al. (33). Rabbit antisera specific for the heavy chains of mouse IgG1 and IgG3 were purchased from Litton Bionetics, Inc. (Kensington, MD). We tested the specificity of these reagents by showing that they developed reverse PFC only of the Ig class(es) shown to be present by radioimmunoassay (RIA) analysis (as described below). PFC assays of cells in limiting dilution cultures were performed after 6 d of culture. Wells with two or more PFC were scored as positive. We analyzed the data according to Poisson statistics as described by Lefkovits and Waldmann (34).

*RIA for Secreted Ig.* 25–50 μl of SN from 6–7 d limiting dilution cultures were assayed for secreted Ig by a solid phase RIA as previously described (35). Briefly, plates were coated with affinity-purified rabbit anti-mouse Ig; secreted IgG in the SN was detected using affinity-purified $^{125}$I-RAMγ1 or $^{125}$I-RAMγ3. These reagents had <1% cross-reactivity with other heavy and light chains. Purified myeloma or hybridoma proteins were used as standards for quantitation. In some experiments, limiting dilution cultures were assayed by the reverse PFC assay. We used an RIA for Ig secretion in the SN from the same cultures to determine the correlation of the two assays. Out of 150 wells assayed for IgG1, 91% gave the same results in both assays; 5% were PFC-positive, but RIA negative, and 4% were negative for PFC, but positive in RIA. Out of 96 wells assayed for IgG3, 90% gave the same result in each assay; 6% were positive for PFC, but negative in RIA and 4% were RIA-positive, but PFC-negative. Correlation of the reverse PFC and RIA data showed that 1 PFC was equivalent to 0.2 ng of secreted IgG (1 ng/ml). Wells with ≥2 PFC were scored as positive; ≥2 ng/ml IgG was considered positive in the RIA. The number of cpm corresponding to 2 ng/ml was always greater than three standard deviations (SD) above the mean cpm obtained from thymus control groups. Since there was no more than a 2% difference between the number of positive wells using the reverse PFC assays and RIA, we used data from both assays to calculate the frequencies of IgG-secreting B cells. Precursor frequencies were calculated by the method of maximum likelihood (36).

The average clone size, $C$, of IgG-secreting B cells was calculated according to the following formula: $C = (\Sigma\text{response})/(U \times W_T)$, where $U$ is the number of precursors per well, $W_T$ is the total number of wells assayed and Σresponse is the total response (PFC or ng/ml IgG) from all wells assayed.

*Sorting of LPS-stimulated Cells.* B cells were cultured with LPS for 2–3 d, then washed, and the viable cells were separated on Ficoll-Hypaque (density, 1.09 g/cc) (37). Cells were treated with F(ab′)$_2$ RAMγ and FITC-GARIg (38). Labeled cells were analyzed with a fluorescence-activated cell sorter (FACS) III (B-D FACS Systems, Becton, Dickinson and Co., Sunnyvale, CA). Large cells were sorted directly into microtiter trays containing medium, thymus filler cells ($3 \times 10^6$/ml), LPS (20 μg/ml), and/or PK 7.1 SN. For selection of sIgG$^-$ cells, the 10–15% brightest (positive) cells were excluded. However, only 2–4% of cells gave staining above background levels. For each subset of cells sorted, two different limiting concentrations of B cells were plated to determine precursor frequencies. We measured the response by RIA after 4 d of culture (a total of 6–7 d of culture), and the data were analyzed according to Poisson statistics (34).

*Replicate Cultures: Procedure and Data Analysis.* In some experiments, limiting dilution cultures were set up with $6 \times 10^6$ thymus cells/ml, 20 μg/ml LPS, and a concentration of splenic B cells that gave ~50% wells responding at the end of the culture. These cultures were divided in half after 3 d of incubation and were restimulated with either LPS or LPS plus PK 7.1 SN. After a total of 7 d of incubation, the replicate cultures were assayed by RIA for secreted IgG3 or IgG1, respectively. The correlation of positive and negative wells in replicates was determined. We used a two-tailed Fisher's exact test (39) to find the probability of obtaining each set of data, assuming independent responses in each replicate. This probability determines the significance of the correlation observed. The correlation coefficient ($r_n$) was also calculated as described for dichotomous nominal scale data (40). $r_n$ is a measure of the degree of correlation and is independent of the level of significance of the data ($P$ value). Therefore, it is necessary that the $P$ value be significant; if it is, the $r_n$ value then indicates how strong the correlation is. In addition, two sets of

TABLE I
*Analysis of Data*

| | Replicate | | Percent of total wells tested |
|---|---|---|---|
| | 1 | 2 | |
| Perfect positive correlation* | | | |
| a. | + | + | 50 |
| b. | + | − | 0 |
| c. | − | + | 0 |
| d. | − | − | 50 |
| | | Total: | $n$ |
| No correlation‡ | | | |
| a. | + | + | 25 |
| b. | + | − | 25 |
| c. | − | + | 25 |
| d. | − | − | 25 |
| | | Total: | $n$ |

* For these data, $r_n = 1.0$, $P < 0.001$.
‡ For these data, $r_n = 0$, $P > 0.9$ (for 48 wells).

data can be compared by chi-square analysis of heterogeneity (41), which determines the probability that the two sets of data are the same. For two sets of replicate cultures in which 50% of the wells were positive in each set, the two extremes of possible results (perfect positive correlation vs. no correlation) are presented in Table I. There is also the possibility of negative correlation ($r_n = -1.0$), but none of the observed data fell into this category. $r_n$ is calculated as follows: $r_n = [(a + d) - (b + c)]/n$, where $a$–$d$ represent respective rows of data in Table I.

In conclusion, it should be stressed that the only valid comparison within an experiment is the comparison with the internal control. Comparisons between experiments must be based on the $r_n$ values, and not the percentage of positive or negative wells in each experiment. Furthermore, the $r_n$ values are only valid if the $P$ values are significant (<0.01).

## Results

*Effect of T Cell–derived BCDFγ-containing SN on the Precursor Frequency and Clone Size of IgG-secreting Cells.* Previous studies (16, 23) indicated that in bulk cultures of LPS-stimulated splenic B cells IgG1 secretion was increased ~10-fold and IgG3 secretion was decreased 3-fold after BCDFγ-containing PK 7.1 SN was added. A change in the precursor frequency and/or size of IgG-secreting clones might explain these observations.

To distinguish between these possibilities, we titrated B cells in limiting dilution cultures. The cells were stimulated with LPS and, after 1 d, PK 7.1 SN was added. 5 d later, IgG1 and IgG3 secretion were determined by reverse PFC assay or by RIA. Figs. 1 and 2 show the effects of PK 7.1 SN on the precursor frequencies of IgG1- and IgG3-secreting cells, respectively. As calculated from the data in these figures, PK 7.1 SN caused a 16-fold increase in the precursor frequency of IgG1-secreting cells and a 6-fold decrease in the precursor frequency of IgG3-secreting cells as compared with cells cultured with LPS alone. PK 7.1 SN had no effect on the clone size of IgG3-secreting cells, but increased

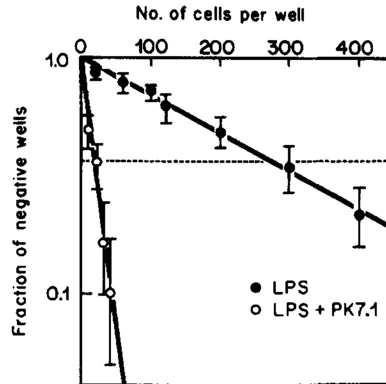


FIGURE 1. BCDFγ-containing PK 7.1 SN induces an increase in precursor frequency of IgG1-secreting cells. Splenic B cells were cultured at limiting dilution in the presence of LPS (●) or LPS and PK 7.1 SN (○) for 6 d. IgG1 secretion was determined by reverse PFC and RIA. Results from four experiments were pooled; each point represents 96–335 wells. Error bars show the 95% confidence limits of each point. The following frequencies ($1/n$) (range) were obtained: for LPS, 272 (249–297); LPS + PK 7.1 SN, 17.1 (15.1–19.4).

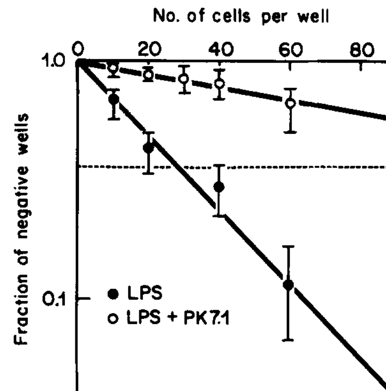


FIGURE 2. BCDFγ-containing PK 7.1 SN induces a decrease in precursor frequency of IgG3-secreting cells. Limiting dilution cultures were set up as described in the legend of Fig. 1. IgG3 secretion was determined by reverse PFC and RIA. Results from three experiments were pooled; each point represents 60–156 wells. Error bars show the 95% confidence limits of each point. Precursor frequencies ($1/n$) (range) of IgG3-secreting B cells were: in the presence of LPS (●) 27.5 (24.6–30.9) or of LPS and PK 7.1 SN (○) 161 (123–211).

the clone size of IgG1-secreting cells 2.8-fold (Table II). Thus, the increased levels of IgG1 secretion induced by the addition of PK 7.1 SN to the limiting dilution cultures are due predominantly to an increase in the precursor frequency of IgG1-secreting cells. The suppression of IgG3 secretion, however, is caused solely by a decrease in precursor frequency of IgG3-secreting cells.

*BCDFγ-containing SN Induce IgG1 Secretion of sIgG⁻ LPS Blasts.* Of importance in understanding the mechanism of action of BCDFγ is whether BCDFγ affects cells before or after expression of sIgG. Our previous investigations (16, 25) indicate that BCDFγ does not bind to sIgG and does not act on cells that were $sIgG^+$ before LPS activation. However, LPS activation induces expression of sIgG (18, 26, 42). Thus, we determined whether cells responding to BCDFγ

TABLE II

*Effect of BCDFγ-containing PK 7.1 SN on the Clone Size of IgG-secreting B Cells**

| Isotype secreted | Number of IgG-secreting cells per clone in the presence of:‡ | | Change in clone size induced by PK 7.1 SN |
|---|---|---|---|
| | LPS | LPS + PK 7.1 SN | |
| IgG3 | 17.3 ± 3.1 | 19.7 ± 2.1 | None |
| IgG1 | 18.9 ± 1.6 | 52.3 ± 6.0 | 2.8× |

* B cells were plated in limiting dilution cultures as described in Materials and Methods and Fig. 1. Secreted IgG was measured by RIA and the data were calculated from experiments shown in Figs. 1 and 2. The mean ± SEM of three to six separate determinations from groups of 48 wells is shown.

‡ The clone size, $C$, was calculated according to the formula: $C = \Sigma\text{Response}/U \times W_T$, where $W_T$ is the total number of wells assayed and $U$ is the number of precursors per well. One PFC was shown to be equivalent to 1 ng/ml of secreted IgG (see Materials and Methods).

TABLE III

*Secretion of IgG1 by Total and sIgG⁻ B Cell Blasts Stimulated With BCDFγ-containing PK 7.1 SN**

| Exp. | Precursor frequency of IgG1-secreting cells (1/n) | |
|---|---|---|
| | Total blasts | $sIgG^-$ blasts‡ |
| 1 | 143 | 92 |
| 2 | 217 | 222 |
| 3 | 130 | 101 |
| Mean ± SEM | 163 ± 27 | 138 ± 42 |

* Splenic B cells were cultured with LPS (20 μg/ml) for 2 (Exp. 3) or 3 d (Exps. 1 and 2) and stained for sIgG. Total blasts or $sIgG^-$ blasts were sorted on a FACS III into microtiter trays for limiting dilution analysis. After four more days of culture with PK 7.1 SN, secretion of IgG1 was determined by RIA and the precursor frequency determined by Poisson statistics.

‡ Blasts had a mean forward light scatter on the FACS III of approximately twice that of the small cell population.

were $sIgG^+$ or $sIgG^-$ after 3 d of LPS activation. We then compared the precursor frequency of IgG1-secreting cells in $sIgG^-$ LPS blasts with the frequency in total LPS blasts when both cell populations were cultured with PK 7.1 SN (it should be stressed that virtually all 3-d LPS blasts were $sIgM^+$, confirming earlier studies [26, 42]). Table III shows that the frequency with which clones of IgG1-secreting cells arose was similar in both cell populations. Thus, most cells which secrete IgG1 after 7 d of culture did not express sIgG after 3 d of activation with LPS.

*Replicate Culture Experiments.* Although the experiments described in the next two sections had different aims, the procedures used were similar for both. Cultures of splenic B cells were set up at limiting dilution, stimulated with LPS, then, after 3 d incubation, divided into two replicates. The replicates were incubated 4 d more, then assayed for IgG secretion. The time interval before the cultures were divided was sufficiently long to allow the responding clones to

have expanded to a point such that splitting resulted in a measurable response in both halves. We had to divide cultures early enough, however, that the addition of PK 7.1 SN would still induce an easily measurable IgG1 response (delaying the addition of PK 7.1 SN reduces the magnitude of the IgG1 response). Because the growth of responding clones is asynchronous, perfect correlation between the responses in the two replicate sets of cultures was not expected, and statistical methods were required to evaluate the data (see Materials and Methods).

Preliminary experiments established that day 3 was the earliest point cultures could be split and still give highly significant ($P < 0.01$) correlation of responses from identically treated replicates. When PK 7.1 SN was added on day 3, the IgG1 precursor frequency was ~50% of that obtained when the SN was added on day 1.

The number of splenic B cells per well was chosen so that ~50% of the wells were positive, i.e., there was an average of less than one precursor per well. It is easier to show significant correlation with even lower proportions of positive wells, but we were restricted in the number of cultures that could be set up by the availability of PK 7.1 SN, and lower proportions of positive wells would have required testing larger numbers of cultures.

There was some variation in both the proportion of positive wells (apparently due to variation in the response of the cells from different groups of mice) and the degree of correlation obtained in individual experiments. Therefore, every experiment contained an internal control in which both replicates were stimulated with LPS and assayed for IgG3. Furthermore, it should be noted that even when wells scored negative for IgG secretion, 80–95% of the wells in all experiments were positive for IgM secretion, demonstrating that negative wells contained growing, IgM-secreting cells, rather than an absence of cells.

*Segregation of the Precursors of IgG1- and IgG3-secreting Cells.* The enhancement of IgG1 secretion and the suppression of IgG3 secretion induced by PK 7.1 SN suggested that the SN was influencing cells to switch from IgG3 to IgG1. Alternatively, the SN could increase IgG1 and decrease IgG3 secretion of two different precursor populations. This might mean that the lymphokines causing these effects are different.

Because PK 7.1 SN caused suppression of the IgG3 response (Fig. 2), we could not directly determine whether the same precursors gave rise to IgG1- and IgG3-secreting cells. Instead, replicate cultures were prepared as described, after 3 d stimulation with LPS. One set of cultures was stimulated with only LPS and assayed for IgG3 secretion, while the other replicate set was stimulated with LPS plus PK 7.1 SN and assayed for IgG1 secretion. Wells giving IgG3 responses were correlated with wells giving IgG1 responses (Table IV, protocol *B*). In control cultures, both replicates were restimulated with LPS and assayed for IgG3 secretion (Table IV, protocol *A*). The control cultures showed a highly significant correlation of the IgG3 responses ($P < 0.001$), and established the degree of correlation ($r_n = 0.672$) that would be expected in Table IV *B* if IgG1- and IgG3-secreting cells were derived from the same precursors. The data in Table IV *B* show that there was only a low degree of correlation ($r_n = 0.167$) of IgG3 and IgG1 responses, indicating that most IgG3 and IgG1 precursors are

TABLE IV

*Segregation of Precursors of IgG1- and IgG3-secreting B Cells in Replicate Cultures**

| Protocol | Pattern of IgG secretion in replicate cultures | | Cultures showing pattern of IgG secretion | | Significance | Correlation ($r_n$) |
|---|---|---|---|---|---|---|
| | | | No. of wells | Percent of total | | |
| | LPS | | | | | |
| | IgG3 | IgG3 | | | | |
| A‡ | + | + | 101 | 53 | | |
| | + | − | 15 | 7.9 | $P < 0.001$ | 0.672 |
| | − | + | 16 | 8.5 | | |
| | − | − | 57 | 30 | | |
| | LPS + PK 7.1 | LPS | | | | |
| | IgG1 | IgG3 | | | | |
| B‡ | + | + | 55 | 29 | | |
| | + | − | 22 | 11 | $P = 0.004$ | 0.167 |
| | − | + | 58 | 30 | | |
| | − | − | 57 | 30 | | |

* Limiting dilution cultures were set up with $6 \times 10^6$ thymus cells/ml, 20 μg/ml LPS, and 20 splenic B cells/well. After 3 d in culture, two replicate cultures were set up from each limiting dilution well. In protocol *A* (internal control), both replicate cultures were restimulated with LPS. After 4 d, the SN were assayed for IgG3 by RIA and the correlation of positive and negative replicate cultures determined. In protocol *B*, one replicate culture was restimulated with LPS and the other replicate culture with LPS plus PK 7.1 SN. The SN from the replicate cultures restimulated with LPS were assayed for IgG3 and the SN from the replicate cultures stimulated with LPS plus PK 7.1 SN were assayed for IgG1 by RIA. The correlation of the IgG1 with the IgG3 responses in the replicate cultures was determined and its significance calculated by Fisher's two-tailed exact test.

‡ By chi-square analysis, the data from protocols *A* and *B* are significantly different ($P < 0.001$).

independent of each other. This low correlation was, however, significant ($P = 0.004$). A small proportion of clones probably give rise to both IgG1- and IgG3-secreting cells.

Since the majority of IgG1-secreting clones do not arise from IgG3-secreting clones, it follows that PK 7.1 SN does not induce a switch from IgG3 to IgG1 secretion. Rather, the suppression of IgG3 and enhancement of IgG1 secretion are separate effects, possibly caused by distinct components of PK 7.1 SN. IgG3 secretion was also determined in the cultures that had been restimulated with LPS and PK 7.1 SN (Table V). In six separate experiments, two of which are shown in Table V, suppression of the IgG3 response, caused by the addition of PK 7.1 SN, varied from 12 to 38% (data not shown). Although this degree of suppression was substantially less than that seen when PK 7.1 SN was added on the first day of culture (Fig. 2), we could not determine whether it would bias the results. Thus, there could be preferential suppression of IgG3 secretion in either the IgG1-positive or IgG1-negative wells. Such suppression would result in a misleading correlation score. However, the data in Table V are similar to the data obtained when the correlation was analyzed in replicate cultures (Table IV *B*). The degree of correlation ($r_n$) of IgG1 and IgG3 responses was slightly

TABLE V

*Segregation of Precursors of IgG1- and IgG3-secreting B Cells in the Same Cultures**

| Exp. | Pattern of IgG secretion in wells containing LPS + PK 7.1 SN | | Cultures showing pattern of IgG secretion | | Significance | Correlation ($r_n$) |
|---|---|---|---|---|---|---|
| | | | No. of wells | Percent of total | | |
| | IgG1 | IgG3 | | | | |
| 1[‡] | + | + | 55 | 29 | | |
| | + | − | 22 | 11 | $P < 0.001$ | 0.292 |
| | − | + | 46 | 24 | | |
| | − | − | 69 | 36 | | |
| | IgG1 | IgG3 | | | | |
| 2[§] | + | + | 63 | 33 | | |
| | + | − | 26 | 14 | $P < 0.001$ | 0.302 |
| | − | + | 41 | 21 | | |
| | − | − | 62 | 32 | | |

* See footnotes to Tables IV and VI for experimental details. IgG3 secretion was determined in cultures that had received LPS + PK 7.1 SN after division of culture on day 3, rather than in the replicates that received only LPS; i.e., the same wells were analyzed for both IgG1 and IgG3.

‡ Data are taken from experiments in Table IV, protocol *B*. The precursor frequency of IgG3-secreting cells was suppressed 21% in replicates receiving LPS + PK 7.1 SN, compared with replicates receiving LPS alone.

§ Data from experiments in Table VI, protocol *B*. The precursor frequency of IgG3-secreting cells was suppressed 32% in the replicates receiving LPS + PK 7.1 SN, compared with replicates receiving LPS alone.

higher in Table V, as would be expected based on technical considerations of analyzing the same wells for IgG1 and IgG3, rather than replicate wells (as in Table IV). In analyzing supernatants from the same wells, the degree of correlation that would be expected if IgG1 and IgG3 precursors were the same is 1.0, rather than an experimentally determined control value, such as that shown in Table IV *A*. Thus, the conclusion reached from the data shown in Table IV *B* is further supported. The majority of IgG3- and IgG1-secreting clones arise from separate precursors, with a small proportion of precursors giving rise to both IgG3- and IgG1-secreting progeny.

*LPS-stimulated B Cells Are Committed to IgG1 Secretion Before Addition of BCDF$\gamma$-containing SN.* As illustrated in Table IV *A*, when, after 3 d in culture, LPS-stimulated clones destined to secrete IgG3 were divided in half, a high proportion (86%) produced IgG3 in both halves after 7 d. This result implies that, after 3 d of stimulation, there were sufficient numbers of cells already committed to IgG3 secretion such that each half would give an identical response 4 d later. A similar protocol was used to determine whether clones destined to secrete IgG1 were already committed to this pathway before addition of PK 7.1 SN, or whether addition of PK 7.1 induced a commitment to IgG1 secretion.

To address this issue, B cells were plated in limiting dilution cultures in the presence of LPS, and replicate cultures were prepared 3 d later. Cells in both replicas received PK 7.1 SN, and IgG1 secretion was assayed 4 d later. Control

TABLE VI
*Commitment of B Cells to IgG1 Secretion Before the Addition of BCDFγ-containing PK 7.1 SN**

| Protocol | Pattern of IgG secretion in replicate cultures | | Cultures showing pattern of IgG secretion | | Significance | Correlation ($r_n$) |
|---|---|---|---|---|---|---|
| | | | No. of wells | Percent of total | | |
| | LPS | | | | | |
| | IgG3 | IgG3 | | | | |
| A[‡] | + | + | 75 | 39 | | |
| | + | − | 21 | 11 | $P < 0.001$ | 0.583 |
| | − | + | 19 | 10 | | |
| | − | − | 77 | 40 | | |
| | LPS + PK 7.1 | | | | | |
| | IgG1 | IgG1 | | | | |
| B[‡] | + | + | 70 | 37 | | |
| | + | − | 29 | 15 | $P < 0.001$ | 0.466 |
| | − | + | 22 | 12 | | |
| | − | − | 70 | 37 | | |

* Limiting dilution cultures were set up (as described in Table IV) in the presence of LPS and, after 3 d in culture, two replicate cultures were set up from each limiting dilution well. In protocol *A* (internal control), 20 splenic B cells were cultured per well. Replicate cultures were restimulated with LPS and assayed for IgG3 secretion by RIA after four additional days. In protocol *B*, 30 splenic B cells were cultured per well. Replicate cultures were restimulated with LPS plus PK 7.1 SN and, after four more days, the culture SN were assayed for IgG1 by RIA. The correlation of positive and negative replicate cultures was determined.
‡ By chi-square analysis, the data from protocols *A* and *B* are not significantly different ($P = 0.24$).

cultures that were stimulated only with LPS and assayed for IgG3 were included in each experiment.

We reasoned that if cultures were already committed to IgG1 secretion before the addition of PK 7.1, the correlation of IgG1 responses in the replicate cultures would be similar to that obtained with the IgG3 responses. On the other hand, if PK 7.1 SN induces a commitment to IgG1 secretion, then there should be no correlation of IgG1 responses in the replicates, since commitment occurs after the cultures are split. The results presented in Table VI show that the correlation of both IgG3 (Table VI, protocol *A*) and IgG1 (Table VI, protocol *B*) responses was highly significant ($P < 0.001$) and that the degree of correlation of IgG1 responses ($r_n = 0.466$) was similar to that of IgG3 responses ($r_n = 0.583$). Thus, clones stimulated by LPS are committed to IgG1 secretion before addition of PK 7.1 SN. However, PK 7.1 SN is needed to induce IgG1 secretion in the committed B cells.

## Discussion

Four major findings have emerged from these studies: (*a*) PK 7.1 SN induces a profound increase (16-fold) in the precursor frequency and a modest increase (2.8-fold) in the clone size of IgG1-secreting B cells; (*b*) cells responding to PK

7.1 SN lack sIgG 3 d after LPS activation; (*c*) the vast majority of IgG1-secreting cells are derived from precursors that are different from those giving rise to IgG3-secreting cells; and (*d*) precursors of IgG1-secreting clones are committed to IgG1 secretion before the addition of PK 7.1 SN. These committed cells lack sIgG and do not secrete IgG1 unless they are cultured with PK 7.1 SN.

The increase in precursor frequency of IgG1-secreting cells induced by PK 7.1 SN suggests that this differentiation step is a directed process. Thus, BCDF$\gamma$ in PK 7.1 SN acts as a differentiation factor. Since PK 7.1 SN also contains growth factors (31), the modest increase in clone size of IgG1-secreting cells might be caused by such factors.

The finding that PK 7.1 SN acts on $sIgG^-$ precursor cells extends the results of Isakson et al. (16), who reported that the target cell for BCDF$\gamma$-containing SN does not bear sIgG before activation with LPS. It was possible, however, that PK 7.1 SN acts on cells that acquire sIgG as a result of LPS stimulation. Indeed, Coutinho et al. (18) have described a maturation factor that induces IgG1 secretion in cells already expressing sIgG1. In contrast, in our experiments, removal of $sIgG^+$ cells after LPS stimulation did not decrease the IgG1 response. Hence, PK 7.1 SN appears to differ from the lymphokines described by Coutinho et al. (18) since it acts on cells that are $sIgG^-$ after 3 d of LPS stimulation.

The data in this report also demonstrate that the majority of IgG1-secreting cells do not arise from precursors of IgG3-secreting cells, but rather, from an independent set of precursors. This suggests that most cells switch directly from IgM to IgG1, and few from IgM to IgG3 to IgG1.

Finally, we have shown that cells are committed to IgG1 secretion before the addition of PK 7.1 SN. We envision two alternative pathways of differentiation during which commitment could occur (see Fig. 3). In the first pathway, receptors for BCDF$\gamma$ appear before DNA rearrangement and transcription of $C\gamma_1$ genes; interaction of BCDF$\gamma$ with its receptor induces rearrangement and transcription. In this pathway, commitment is manifested by the expression of BCDF$\gamma$ receptors. In the second pathway, DNA rearrangements occur before the appearance

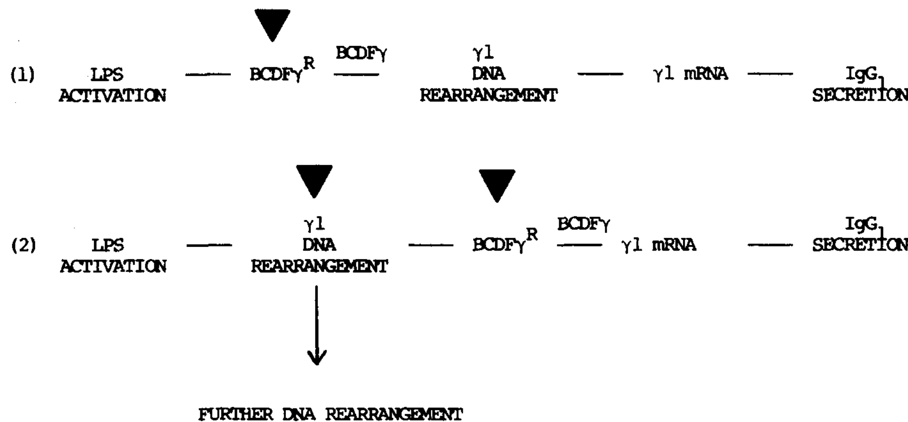


FIGURE 3. Diagrammatic representation of two alternative pathways for differentiation to IgG1 secretion. (▼) The stage at which commitment to IgG1 secretion could occur. $BCDF\gamma^R$ is the receptor for BCDF$\gamma$.

of BCDF$\gamma$ receptors. Rearrangements would determine commitment if, subsequently, receptors for BCDF$\gamma$ are expressed, and binding of BCDF$\gamma$ to its receptor take place. However, DNA rearrangements might continue for heavy chain constant region ($C_H$) genes 3′ to $C\gamma_1$, with concomitant expression of receptors for the lymphokines that induce secretion of other isotypes. In this case, the initial rearrangement for $C\gamma_1$ expression would not necessarily constitute commitment to IgG1 secretion. To distinguish between these possibilities, it would be helpful to raise antibodies to the BCDF$\gamma$ receptor, so that cells bearing the receptor can be purified and their DNA analyzed for the relevant rearrangements.

## Summary

To gain insight into how T cell–derived lymphokines induce the secretion of IgG in activated B cells, we performed a limiting dilution analysis, using murine splenic B cells incubated with lipopolysaccharide (LPS) and a T cell–derived B cell differentiating factor for IgG (BCDF$\gamma$)-containing supernatant (SN). The results of this analysis indicate that such a SN induces a marked increase in the precursor frequency of IgG1-secreting cells and a modest increase in clone size. The precursors lack surface IgG and are committed to the differentiation pathway for IgG1 secretion after LPS activation, but before the addition of BCDF$\gamma$-containing SN. The majority of IgG1-secreting clones arise independently from precursors of cells that secrete IgG3. Taken together, these results indicate that BCDF$\gamma$ directs differentiation of activated B cells to IgG1 secretion.

We thank Drs. Peter Isakson and Ellen Puré for helpful discussions regarding the experimental design and the manuscript. We thank Ms. C. Wiles, Ms. L. Trahan, Ms. F. LaMontagne, Ms. S. Gorman, Mr. Y. Chinn, Ms. R. Baylis, and Mr. W. Muller for technical assistance, and Ms. G. A. Cheek for secretarial assistance.

*Received for publication 9 April 1984 and in revised form 16 August 1984.*

## References

1. Honjo, T., and I. Kataoka. 1978. Organization of immunoglobulin heavy chain genes and allelic deletion model. *Proc. Natl. Acad. Sci. USA.* 75:2140.
2. Cory, S., and J. M. Adams. 1980. Deletions are associated with somatic rearrangement of immunoglobulin heavy chain genes. *Cell.* 19: 37.
3. Rabbitts, T. H., A. Forster, W. Dunnick, and D. L. Bentley. 1980. The role of gene deletion in the immunoglobulin heavy chain switch. *Nature (Lond.).* 283:351.
4. Yaoita, Y., and T. Honjo. 1980. Deletion of immunoglobulin heavy chain genes from expressed allelic chromosome. *Nature (Lond.).* 286:850.
5. Hurwitz, J. L., and J. J. Cebra. 1982. Rearrangements between the immunoglobulin heavy chain gene $J_H$ and $C\mu$ region accompany normal B lymphocyte differentiation in vitro. *Nature (Lond.).* 299:742.
6. Radbruch, A., and F. Sablitzky. 1983. Deletion of $C\mu$ genes in mouse B lymphocytes upon stimulation with LPS. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:1929.
7. Taylor, R. B., and H. H. Wortis. 1968. Thymus dependence of antibody: variation with dose of antigen and class of antibody. *Nature (Lond.).* 220:927.
8. Torrigiani, G. 1972. Quantitative estimation of antibody in the immunoglobulin

classes of the mouse. II. Thymus dependence of the different classes. *J. Immunol.* 108:161.

9. Braley-Mullen, H. 1974. Regulatory role of T cells in IgG antibody formation and immune memory to type III pneumococcal polysaccharide. *J. Immunol.* 113:1909.
10. Rosenberg, Y. J., and J. M. Chiller. 1979. Ability of antigen-specific helper cells to effect a class-restricted increase in total Ig-secreting cells in spleens after immunization with the antigen. *J. Exp. Med.* 150:517.
11. Slack, J., G. P. Der-Balian, M. Nahmn, and J. M. Davie. 1980. Subclass restriction of murine antibodies. II. The IgG plaque-forming cell response to thymus-independent type 1 and type 2 antigens in normal mice and mice expressing an X-linked immunodeficiency. *J. Exp. Med.* 151:853.
12. Mongini, P. K. A., K. E. Stein, and W. E. Paul. 1981. T cell regulation of IgG subclass antibody production in response to T-independent antigens. *J. Exp. Med.* 153:1.
13. Kawanishi, H., L. E. Salzman, and W. Strober. 1983. Mechanisms regulation IgA class-specific immunoglobulin production in murine gut-associated lymphoid tissues. I. T cells derived from Peyer's patches that switch sIgM B cells to sIgA B cells in vitro. *J. Exp. Med.* 157:433.
14. Kishimoto, T., and K. Ishizaka. 1975. Immunologic and physical properties of enhancing soluble factors for IgG and IgE antibody responses. *J. Immunol.* 114:1177.
15. Suemura, M., J. Yodoi, M. Hirashima, and K. Ishizaka. 1980. Regulatory role of IgE-binding factors from rat T lymphocytes. I. Mechanism of enhancement of IgE response by IgE potentiating factor. *J. Immunol.* 140:895.
16. Isakson, P. C., E. Puré, E. S. Vitetta, and P. H. Krammer. 1982. T cell–derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. *J. Exp. Med.* 155:734.
17. Mayer, L., S. M. Fu, and H. G. Kunkel. 1982. Human T cell hybridomas secreting factors for IgA-specific help, polyclonal B cell activation, and B cell proliferation. *J. Exp. Med.* 156:1860.
18. Coutinho, A., S. Pettersson, E. Routh, and L. Forni. 1983. Immunoglobulin C gene expression. IV. Alternative control of $IgG_1$-producing cells by helper cell-derived B cell-specific growth or maturation factors. *Eur. J. Immunol.* 13:269.
19. Kawanishi, H., L. Saltzman, and W. Strober. 1983. Mechanisms regulating IgA class-specific immunoglobulin production in murine gut-associated lymphoid tissues. II. terminal differentiation of postswitch sIgA–bearing Peyer's patch B cells. *J. Exp. Med.* 158:649.
20. Bergstedt-Lindqvist, S., P. Sideras, H. R. MacDonald, and E. Severinson. 1984. Regulation of Ig class secretion by soluble products of certain T cell lines. *Immunol. Rev.* 78:25.
21. Kearney, J. F., and A. R. Lawton. 1975. B lymphocyte differentiation induced by LPS. I. Generation of cells synthesizing four major Ig classes. *J. Immunol.* 115:671.
22. Andersson, J., A. Coutinho, and F. Melchers. 1978. The switch from IgM to IgG secretion in single mitogen-stimulated B-cell clones. *J. Exp. Med.* 147:1744.
23. Jones, S., Y.-W. Chen, P. C. Isakson, J. Layton, E. Pure, C. Word, P. Krammer, P. Tucker, and E. S. Vitetta. 1983. Effect of T cell-derived lymphokines containing BCDF$\gamma$ on $\gamma$-specific mRNA in murine B cells. *J. Immunol.* 131:3049.
24. Isakson, P. C., E. Pure, E. S. Vitetta, and P. H. Krammer. 1982. T cell-derived B cell differentiation factors (BCDF): definition of BCDF$\mu$ and BCDF$\gamma$. *UCLA Symp. Mol. Cell. Biol.* 24:391.
25. Vitetta, E. S., K. Brooks, Y.-W. Chen, P. Isakson, S. Jones, J. Layton, G. C. Mishra, E. Pure, E. Weiss, C. Word, D. Yuan, P. Tucker, J. W. Uhr, and P. H. Krammer.

1984. T cell-derived lymphokines inducing IgM and IgG secretion in activated murine B cells. *Immunol. Rev.* 78:137.

26. Yuan, D., and E. S. Vitetta. 1983. Structural studies of cell surface and secreted IgG in LPS-stimulated murine B cells. *Mol. Immunol.* 20:367.
27. Word, C., and W. M. Kuehl. 1981. Expression of surface and secreted $IgG_{2a}$ by a murine B lymphoma before and after hybridization to myeloma cells. *Mol. Immunol.* 18:311.
28. Gronowicz, E. S., C. A. Doss, F. D. Howard, D. C. Morrison, and S. Strober. 1980. An *in vitro* line of the B cell tumor $BCL_1$ can be activated by LPS to secrete IgM. *J. Immunol.* 125:976.
29. Krammer, P. H., M. Dy, L. Hultner, P. Isakson, U. Kees, M. L. Lohmann-Mathes, F. Marcucci, A. Michnay, E. Pure, A. Schimpl, F. Staber, E. S. Vitetta, and M. Waller. 1982. Production of lymphokines by murine T cells grown in limiting dilution and long term cultures. *In* Isolation, Characterization and Utilization of T Lymphocyte Clones. G. Fathman and F. Fitch, editors. Academic Press, Inc., New York, 253–262.
30. Schimpl, A., and E. Wecker. 1972. Replacement of T cell function by a T cell product. *Nat. New Biol.* 237:15.
31. Pure, E., P. C. Isakson, J. W. Kappler, P. Marrack, P. H. Krammer, E. S. Vitetta. 1983. T cell–derived B cell growth and differentiation factors. Dichotomy between the responsiveness of B cells from adult and neonatal mice. *J. Exp. Med.* 157:600.
32. Pike, B. L., G. Jennings, and K. Shortman. 1982. A simple semi-automated plaque method for the detection of antibody-forming cell clones in microcultures. *J. Immunol. Methods.* 52:25.
33. Gronowicz, E., A. Coutinho, and F. Melchers. 1976. A plaque assay for all cells secreting Ig of a given type or class. *Eur. J. Immunol.* 6:588.
34. Lefkovits, I., and H. Waldmann. 1979. *In* Limiting Dilution Analysis of Cells in the Immune System. Cambridge University Press, Cambridge. 60–66.
35. Isakson, P. C., K. A. Krolick, J. W. Uhr, and E. S. Vitetta. 1980. The effect of anti-immunoglobulin antibodies on the in vitro proliferation and differentiation of normal and neoplastic murine B cells. *J. Immunol.* 125:886.
36. Fazekas de St. Groth, S. 1982. Evaluation of limiting dilution assays. *J. Immunol. Methods.* 49:R11.
37. Parish, C. R., S. M. Kirov, N. Bowern, N. Blanden, and R. V. Blanden. 1974. A one step procedure for separating mouse T and B lymphocytes. *Eur. J. Immunol.* 4:808.
38. Goding, J. W. 1976. Conjugation of antibodies with fluorochromes: modifications to the standard methods. *J. Immunol. Methods* 13:215.
39. Fisher, R. A. 1958. Statistical Methods for Research Workers. Hafner Publishing Co., Inc., New York. 13th ed. p. 96.
40. Zar, J. H. 1974. Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, NJ. p. 245.
41. Zar, J. H. 1974. Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, NJ. p. 63.
42. Bourgeois, A., K. Kitajima, I. R. Hunter, and B. A. Askonas. 1977. Surface immunoglobulins of lipopolysaccharide-stimulated cells. The behavior of IgM, IgD, and IgG. *Eur. J. Immunol.* 7:151.