

THE GENERATION AND REGULATION OF LYMPHOCYTE POPULATIONS

Evidence from Differentiative Induction Systems in Vitro*

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As the cells that are programmed to generate the various sets of immunocompetent lymphocytes progress along their several pathways they pass through discrete phases of differentiation that can be recognized by the selective expression of cell surface components. Detailed investigation of the nature and mechanisms of these differentiative steps has been much facilitated by the development of short-term assays in which some of these phenotypes are induced in vitro (1-4). We present here an account of our findings with induction assays that monitor an early step in T-cell differentiation, marked by acquisition of the surface component Thy-1, and two steps in B-cell differentiation, the first marked by acquisition of complement receptors (CR)¹ (5) and the second by acquisition of the surface component PC (plasma cell antigen) (6).

Materials and Methods

Congenetic α -Thy-1.2 Antiserum [A-Thy-1.1 \times AKR-H-2^b anti-A strain leukemia ASL1]. For use in induction assays this antiserum was preabsorbed with Thy-1.1 thymocytes and leukemia cells until negative in cytotoxicity assays with congenic Thy-1.1 thymocytes. Resultant specific titers against congenic Thy-1.2 thymocytes were $>1:2,000$. Without this preabsorption, unidentified contaminant antibody (possibly murine leukemia virus-related) can give erroneous positive results. Anti-Thy-1.2 antiserum of lower specific titer is inadequate for induction assays. (The same requirements for preabsorption and high specific titer apply equally to the alternative antiserum α -TL, which has given results similar to α -Thy-1 in all respects [7].)

PC Antisera and PC Induction Assay. The same considerations of titer and specificity apply to α -PC serum (DBA/2 \times B6)F₁ anti-BALB/c myeloma MOPC-70A (6), in PC induction assays. This antiserum was preabsorbed with thymus and spleen of PC⁻ strains of mice, including B6, the resulting specific cytotoxicity titer being 1:64 against MOPC-70A cells with guinea pig serum (GPS) as the source of complement, and 1:32 or 1:64 against induced Peyer's patch cells, with rabbit C. We found Peyer's patches to be the richest source of PC-inducible Ig⁺:CR⁺:PC⁻ cells, but their proportion is still too low for a satisfactory induction assay. Therefore we used Peyer's patch

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¹ *Abbreviations used in this paper:* α , anti; B6, C57BL/6; BSA, bovine serum albumin, cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; C, complement; CR, complement receptor; EAC, erythrocyte antibody complement complex; EDTA, ethylene diamine tetraacetate; FcR, Fc receptor; GPS, guinea pig serum; MuLV, murine leukemia virus; PC, plasma cell antigen; PDE, phosphodiesterase; PGE₁, prostaglandin E₁; Poly A:U, polyadenylic-polyuridylic acid; R- α -MIg, rabbit anti-mouse immunoglobulin; SRBC, sheep erythrocytes.

cells from mice stimulated with *Corynebacterium parvum* (1 mg per animal, i.p., 5-7 days before harvest), cleared of dead cells by a two-step bovine serum albumin (BSA) gradient. This yielded 25-30% PC-inducible cells (scored with preabsorbed α -PC serum diluted 1:16, after 2.5-3 h incubation).

Other Antisera. Rabbit anti-mouse immunoglobulin (R- α -MIg) consisted of immunoabsorbent-purified α -MIgM combined with immunoabsorbent-purified α -F(ab)₂ of MIgG (4), used in cytotoxicity procedures at a final concentration of 0.1 mg/ml. Purified 19S fraction of rabbit α -sheep erythrocytes (SRBC) (Cordis Laboratories, Inc., Miami, Fla.) diluted 1:100 was used in the CR assay.

Complement (C). Ample rabbit C is crucial to cytotoxicity assays for induction of cell-surface markers. GPS is generally inadequate. Rabbits were screened for low natural cytotoxicity for thymocytes and high C activity in a system (α -Lyt-2.2) which demands a high level of C. For each pool of C, usually one or two rabbits were selected from 20-30 pretested rabbits.

Thymopoietin (8) and ubiquitin (3) were lyophilized in 1- μ g lots, with 100 μ g BSA to reduce loss from adsorption to glass, and dissolved in RPMI-1640 immediately before use.

Pharmacological Agents. cAMP (adenosine 3':5' monophosphoric acid); DBcAMP (N₆O₂-dibutyryl-adenosine 3':5' monophosphoric acid); theophylline; DL-isoproterenol; L-isoproterenol; DL-propranolol; epinephrine (L-epinephrine); imidazole, carbachol (carbamylocholine chloride); cycloheximide (Sigma Chemical Co., St. Louis, Mo.). We thank Dr. M. Weksler (Cornell Medical College) for providing L-propranolol, Dr. J. Pike (Upjohn Co., Kalamazoo, Mich.) for PGE₁ (prostaglandin E₁), Dr. C. Henney (Johns Hopkins University) for cholera toxin (cholera-enterotoxin) and 8-bromo-cGMP (8-bromo-cyclic guanosine monophosphate), Dr. G. Renoux (Tours-Cedex, France) for poly A:U (polyadenylic-polyuridylic acid), and Dr. C. Galanos (Freiburg) for lipopolysaccharide (endotoxin) and lipid A.

Erythrocyte Antibody Complement Complex (EAC) Indicator Cells. SRBC were washed and suspended in medium 199 (5%); an equal volume of rabbit α -SRBC added; incubated (37°C, 15 min); washed three times and resuspended (5%); added to an equal volume of normal AKR mouse serum (diluted 1:5); further incubated (37°C, 45 min); washed twice, and resuspended in RPMI-1640 (5%).

Density-Gradient Centrifugation to Fractionate the Spleen Cell Population for Enrichment of Inducible Cells (illustrated in Fig. 1). 5-10 \times 10⁷ washed spleen cells (85-95% viable) were pelleted in a 5-ml Beckman cellulose nitrate tube (150 g). The supernate was removed, and the walls of the tube dried. The cells were suspended in 1 ml 35% BSA (Pathocyte 5, lot 21, 22; Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.), overlaid with four 1-ml layers of BSA (29:26:23:10%) as shown in Fig. 1, and the gradient was centrifuged (Beckman model L; SW50.1 rotor; 13,000 rpm; 30 min). Each layer was aspirated in turn through each interface to the midpoint of the next layer, diluted at least 1:15 with RPMI-1640, and the cells washed twice before use in induction assays. Some lots of BSA were unsuitable because they contained >1 μ g/ml endotoxin, itself an inducer (9), and Ficoll proved unsatisfactory because some lots had inducing activity.

The Standard Cell Population for Common use in Thy-1 and CR Induction Assays. The standard cell population used as the common source of cells for prothymocyte and pro-CR⁺ cell assays was the combined B and C layers indicated by the arrows in Fig. 1. To avoid high backgrounds of pre-existing CR⁺ cells in the CR induction assay, we used spleen from *nu/nu* mice, which if healthy have fewer CR⁺ cells than *nu/+* and other phenotypically normal mice (Dr. M. C. Gelfand, personal communication; and M. P. Scheid, unpublished observations). We used *nu/nu* mice of our own colony (bred on a BALB/c background and maintained under conventional conditions), aged 1-2 mo. Spleen cells of normal mice, depleted of CR⁺ cells by sedimentation as rosettes with EAC (5), were used with equal success for preparing the standard cell population when *nu/nu* mice were not available.

General Description of the Induction Assays. The cells (1-5 \times 10⁷ cells/ml RPMI-1640) were distributed into 5-ml plastic tissue culture tubes (BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.). An equal volume of inducer (in RPMI-1640) was added. After incubation for 10 min at 37°C in a waterbath (to equilibrate temperature) the tubes were placed in a humidified 5% CO₂ incubator at 37°C for 2-2.5 h. Afterwards the cells were washed twice in RPMI-1640, resuspended in medium 199 for cytotoxicity assays, or RPMI-1640 for CR assays.

The CR⁺ phenotype for assay of B cells (5) was scored as follows: 0.3 ml EAC (0.5% suspension) was added to 0.3 ml of the induced cell suspension (2×10^6 /ml), and EDTA to 0.01 M, in a Beem plastic electron-microscopy capsule (Ernest F. Fullam, Inc., Schenectady, N.Y.). The suspension was shaken at 37°C in a waterbath and then placed on ice; (the capsules can be held at 4°C overnight without change in the readings). A rosette was defined as a cell binding ≥ 3 EAC. At least 300 cells from each tube were counted, and the proportion of rosetted cells expressed as the percentage of total nucleated cells (rosetted plus nonrosetted). Controls for which due allowance was made in calculating CR-specific complex rosetting and induction indices included: (a) erythrocyte antibody substituted for EAC, and (b) omission of the cells tested for induction.

Controls for Background Induction and for Loss of Surface Markers during Induction Assays. Each assay was controlled for the following three factors: (a) marker-positive cells in the starting population: this was estimated by cytotoxicity assays on the standard cell population immediately before induction assay (Control A). (b) Induction by contaminants: contaminants such as endotoxin in some lots of BSA, encountered during fractionation of spleen cells, produce a background of induced cells after 2 h of culture. This was estimated by control cultures to which no inducer was added (Control B). Control B should equal Control A if there is no background induction. (c) Loss of surface markers: the proportion of cells that register FcR, Ig, or CR, but never Thy-1 or PC, may fall in controls at 37°C without added inducer (Control B). This does not occur at 0°C. Hence Control C (for induction assays of FcR, Ig, and CR) which is the same as Control B but at 0°C. Any test in which controls A, B, and C differed significantly was discarded.

Calculation of Percentages of Cells Induced. The formula for all three markers (Thy-1, CR, PC) was $(a - b)/(100 - b) \times 100$, where a = percent marker-positive cells in the presence of inducer, and b = percent marker-positive cells in the absence of inducer. The values of b in a series of 12 representative tests were 8 (SEM ± 5) for the prothymocyte assay, 13 (SEM ± 5) for the pro-CR⁺ assay, and generally <5 for the PC assay.

Elimination of Thy-1⁺ and Ig⁺ Cell Populations. Washed spleen cells were suspended (2×10^7 /ml) in α -Thy-1.2 (1:50) or α -MIg (1:100); incubated at 4°C for 30 min; washed once; suspended at the original concentration in rabbit serum (C) diluted 1:15; incubated (shaken) for 30 min at 37°C in a waterbath; counted in trypan blue; layered on a two-step (10-35%) BSA gradient; and centrifuged to separate the dead cells. Cytotoxicity assays of the viable cells recovered at the interface showed $>90\%$ to be Thy-1⁻ or Ig⁻, respectively. A control in which antiserum was omitted was always run, to confirm that the pool of C in use still gave negligible cytolysis.

Results

I. *Enrichment of Precursor Cells by Density Gradient Centrifugation; Distribution of Cell Types in the Standard Gradients.* The term prothymocytes (10) is used for the cells in bone marrow and spleen which lack the T-cell surface antigens Thy 1:TL:Lyt but express them when induced to differentiate in vitro (1).

The term pro-CR⁺ cells refers to cells of the B-lymphocyte lineage, in the spleen and to a lesser extent in bone marrow, which do not express CR but do so when induced to differentiate in vitro (granulocytes and macrophages are CR⁺, but form CR rosettes only in the presence of divalent cations [11] which EDTA excludes in our CR assay).

Fig. 1 shows the BSA gradient used and (for A strain spleen) the distribution profiles of: (a) prothymocytes, (b) pre-existing Thy-1⁺ cells, (c) pro-CR⁺ cells, and (d) pre-existing CR⁺ cells. The profiles of all four cell types, shown on the right in Fig. 1, are different from one another, implying that prothymocytes and pro-CR⁺ cells are different inducible populations.

II. *The Kinetics of T-Cell and CR⁺ B-Cell Induction.* Demonstrable induction of Thy-1 and TL expression requires 2 h (1). In a preparatory study we found induction of CR⁺ cells by ubiquitin to be virtually maximal at 2 h in cells

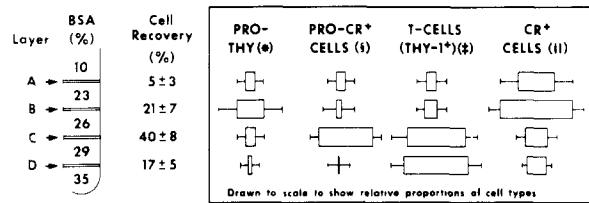


FIG. 1. Distribution of spleen cell types in the standard BSA gradient.

* Prothymocytes: phenotype $TL^{-}:Thy\ 1^{-}$, inducible to express all thymocyte surface markers; § Pro-CR⁺ cells: B-lineage cells inducible to express CR; phenotype $Ig^{+}:Ia^{+}:CR$; ‡ T cells: phenotype $TL^{-}:Thy\ 1^{+}:Lyt^{+}$. ||CR⁺ cells: $Ig^{+}:Ia^{+}:CR^{+}$. Layers B and C combined generally from nu/nu mice, are referred to as the standard cell population for induction (see Materials and Methods). The data shown here are averages for $5-10 \times 10^7$ spleen cells, from A strain mice aged 2-3 mo, on the standard gradient (volume of each of the five layers = 1 ml).

from mice aged 2-3 mo; a longer induction period may be required for cells from younger donors.

Induction does not require presence of inducer throughout the 2-h period. 10 min, the shortest time tested, sufficed for near-maximal registration of induction (at 2 h) by ubiquitin in both assays and by thymopoietin selectively in the T-cell assay. Thus the time required for manifestation of the induced phenotype is much longer than the time required for initiation by either inducer. These data suggest initiation by a surface ligand which triggers intermediary events leading to phenotypic expression. Mediation by cAMP is suggested by reports that its addition causes induction and that pharmacological interference with cAMP metabolism blocks induction, in both assays (2). We tested this further by adding imidazole during induction, in a concentration at which imidazole's known action is to decrease cellular cAMP and increase cGMP, probably by activating cellular cAMP phosphodiesterase (12). Imidazole inhibited induction in both assays completely when added 10 min after the inducer, and partially when added up to 30 min later (Table I). Thus the critical period for cAMP action appears to be the first 30 min of the induction process.

III. *Metabolic Requirement for Induction.* Prothymocyte induction requires cell metabolism; it is prevented by lowering the temperature and by inhibitors of transcription and translation (13). The same is true of pro-CR⁺ B-cell induction; we find no CR induction at 4°C. In both assays, cycloheximide blocked induction completely at concentrations of 2.5 and 10 μ g/ml (which reduce cellular incorporation of [³H]leucine by about 95% [13]), and partially at 0.1 μ g/ml (25% inhibition of [³H]leucine uptake [13]).

IV. *Further Evidence that Prothymocytes and Pro-CR⁺ Cells are Discrete Populations.* Fig. 2 shows: (a) that thymopoietin, throughout its entire range of effective concentration, generates only thymocytes (panels 1 and 2) whereas ubiquitin generates both thymocytes and CR⁺ cells (panels 3 and 4). (b) Induction by ubiquitin is prevented by propranolol (10^{-5} M), a β -adrenergic blocking agent (14), in both assays (panels 3 and 4), whereas induction of prothymocytes by thymopoietin is not prevented by propranolol (panel 1).

If there were a single uniform immediate precursor population capable of differentiating to thymocytes or to CR⁺ B cells, then induction of the population

TABLE I
Suppression of Prothymocyte and Pro-CR⁺ B-Cell Induction by Imidazole

Ubiquitin (0.1 μg/ml)	Imidazole (10 ⁻⁷ M) added after:					Cells induced %			
	0	10	30 min	60	90	Pro-thymocyte assay		Pro-CR ⁺ cell as- say	
						1st Exp.	2nd Exp.	1st Exp.	2nd Exp.
None						0 (Standard)	0 (Standard)	0 (Standard)	0 (Standard)
Present						15	20	16	22
None	+					-1	-4	-3	-3
Present	+					-8*	-5	-3	-4
Present		+				-6	-2	2	-1
Present			+			11	15	6	17
Present				+		16	24	10	15
Present					+	10	19	15	17

* High negative indices presumably reflect imidazole's suppression of the usual low background of cells induced in controls without an added inducer.

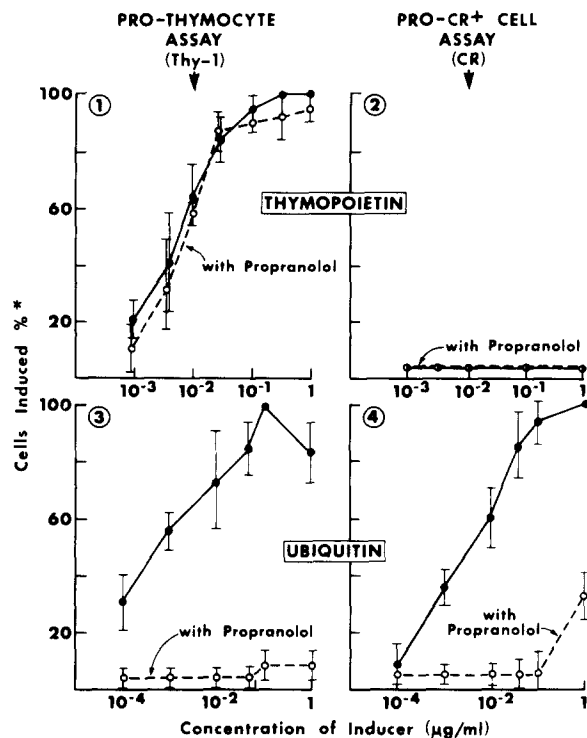


FIG. 2. Effects of propranolol (10⁻⁵ M) on induction by thymopoietin (panels 1 and 2) and ubiquitin (panels 3 and 4) in dual induction assays with the standard cell population. (Mean values for four separate sets.)

* Because the proportion of cells induced varies somewhat according to the cell preparations used in different experiments, the data shown are standardized as percent of maximal induction in the positive experiments, the data shown are standardized as percent of maximal induction in the positive experiments (no inhibitor; optimal concentration of inducer) in each test.

TABLE II
Inductive Selectivity of Thymopoietin and of Ubiquitin, as Indicated by Sequential Induction Assays

Inducer (0.1 $\mu\text{g/ml}$)	First induction		Inducer (0.1 $\mu\text{g/ml}$)	Second induction	
	Cells induced %			Cells induced %*	
	Pro-thymocyte assay	Pro-CR ⁺ cells assay		Pro-thymo- cyte assay	Pro-CR ⁺ cell assay
Thymopoietin	15 \pm 7	<2	Ubiquitin	<2	40 \pm 4
Ubiquitin	16 \pm 5	23 \pm 3	Ubiquitin	<2	35 \pm 5
None	0 (Standard)	0 (Standard)	Ubiquitin	12 \pm 3	25 \pm 5

* Mean \pm SEM for four experiments.

TABLE III
Prothymocyte Induction Assays with R- α -MIg-Depleted Cell Populations

Step 1: Cytolysis	Step 2: Induction Inducer:	Cells induced %*	
		Pro-thymocyte assay	Pro-CR ⁺ cell as- say
R- α -MIg + C	None	0 (Standard)	0 (Standard)
	Thymopoietin (0.5 $\mu\text{g/ml}$)	26 \pm 3	5 \pm 3
	Endotoxin (30 $\mu\text{g/ml}$)	21 \pm 4	-1 \pm 5
C Alone (control)	None	0 (Standard)	0 (Standard)
	Thymopoietin (0.5 $\mu\text{g/ml}$)	22 \pm 3	-3 \pm 2
	Endotoxin (30 $\mu\text{g/ml}$)	19 \pm 6	20 \pm 5

The starting population for step 1 was unfractionated spleen cells, after which the standard cell population was prepared as usual.

* Mean \pm SEM for four experiments.

by thymopoietin, which produces only thymocytes, should leave no cells capable of subsequently being induced to form CR⁺ cells.

Table II shows data for experiments in which the standard cell population was treated with thymopoietin or with ubiquitin (first induction) and then with α -Thy 1 + C to eliminate the thymocytes induced. The cells were then subjected to a second induction, with ubiquitin. There was no further yield of T cells. The higher proportion of CR⁺ cells after the second induction is due to the relative enrichment of CR⁺ cells caused by elimination of the T cells after the first induction. This result is not compatible with generation of thymocytes and CR⁺ cells from the same immediate precursor pool.

Table III shows data for experiments in which pro-CR⁺ cells, which are Ig⁺, were eliminated from the standard cell population by cytolysis with R- α -MIg + C. Subsequent induction revealed no loss of inducible prothymocytes. These data further support the conclusion that prothymocytes and pro-CR⁺ B cells are distinct populations.

V. *Evidence That There are Different Receptors for Thymopoietin and for*

TABLE IV
Receptor Specificity: Effect of Excess Ubiquitin on Sensitivity to Induction by Ubiquitin and by Thymopoietin

Inducer*	Cells induced %‡	
	Pro-thymocyte assay	Pro-CR ⁺ cell assay
Thymopoietin	18 ± 5	1 ± 3
Ubiquitin	1 ± 3	1 ± 2
Thymopoietin + Ubiquitin	15 ± 7	1 ± 4
DBcAMP (1 mM) + Ubiquitin	13 ± 5	10 ± 2

* In a concentration of 0.5 $\mu\text{g/ml}$ for thymopoietin, and 200 $\mu\text{g/ml}$ (predetermined induction-inhibitory concentration) for ubiquitin.

‡ Mean \pm SEM of three experiments.

Ubiquitin. The observations that thymopoietin induces only prothymocytes whereas ubiquitin also induces pro-CR⁺ B cells, and that propranolol, which prevents binding of β -adrenergic agonists (14), blocks induction by ubiquitin but not by thymopoietin (Fig. 2), suggest that the two inducers react with different receptors. Furthermore, high concentrations of ubiquitin do not induce prothymocytes or pro-CR⁺ cells (Table IV, line 2), but this block does not hinder induction of prothymocytes by thymopoietin (line 3), nor of prothymocytes or pro-CR⁺ cells by DBcAMP (line 4).

VI. *Pharmacology of Induction.* The evidence above points to two distinct precursor populations which are precommitted to the thymocyte and B-cell phenotypes, respectively, and to a common intermediary mechanism, probably involving cAMP. Pharmacological evidence supports this interpretation.

CYCLIC NUCLEOTIDES. Because cAMP and DBcAMP are inducers (2), cGMP should have counter-effects if lymphocyte induction conforms to known cAMP-mediated systems (14). Panels 1 and 2 in Fig. 3 give dose-response curves for DBcAMP and show no induction by cGMP (8-bromo cGMP) in either assay over the same entire range of concentrations. But cGMP prevented induction by ubiquitin in both assays when added 10 min beforehand (panels 3 and 4), and so did carbachol (panels 3 and 4), an acetylcholine analog which raises cellular levels of cGMP in lymphocytes (16, 17).

PHARMACOLOGICAL AGENTS AFFECTING CYCLIC NUCLEOTIDE PHOSPHODIESTERASE (PDE) ACTIVITY. Imidazole activates PDE and lowers the ratio of cAMP to cGMP in lymphocytes (12). We tested theophylline and imidazole with optimal and suboptimal concentrations of both thymopoietin and ubiquitin. Theophylline (which at the concentration used, 2×10^{-3} M, had no measurable inductive effect) enhanced the activity of suboptimal concentrations of both thymopoietin and ubiquitin, but suppressed induction by optimal concentrations of both inducers. This suppression is predictable from the dose-response curves for cAMP (Fig. 3, panels 1 and 2) which show inhibition of its own inductive action at high concentration. We also found that inclusion of cAMP with optimal and suboptimal concentrations of either thymopoietin or ubiquitin reproduced these effects of theophylline. In contrast to theophylline, which augments induction, imidazole (10^{-7} M) suppressed induction over the entire response range of

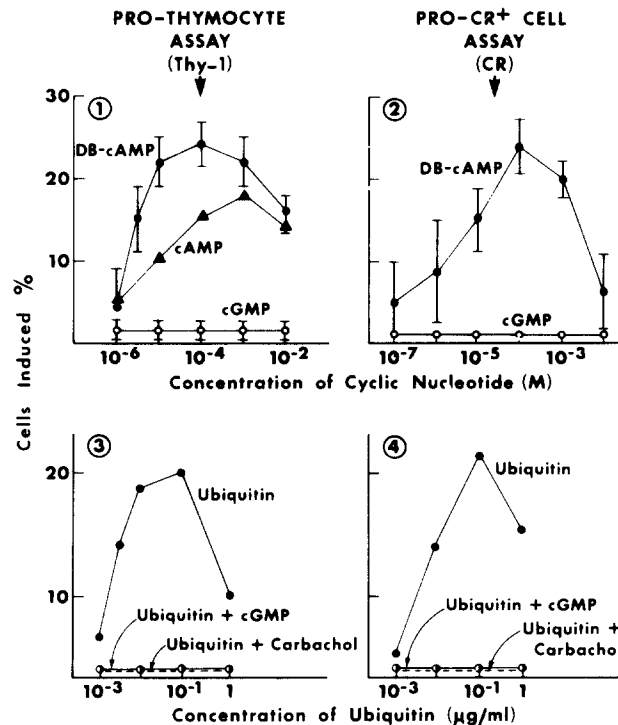


FIG. 3. Cyclic nucleotides. The dose-response curves for induction by DBcAMP and cAMP (panels 1 and 2) show inhibition at high concentrations. There is no induction by cGMP (8-bromo-cGMP) at any concentration (panels 1 and 2), but cGMP (10^{-3} M), and carbachol (10^{-8} M), inhibit induction by ubiquitin if added 10 min beforehand (panels 3 and 4). (Mean values of four separate tests.)

effective concentrations of thymopoietin or ubiquitin. (In higher concentrations, imidazole itself is an inducer, probably for reasons other than its influence on cAMP PDE [12].)

PHARMACOLOGICAL AGENTS ACTIVATING ADENYLATE CYCLASE: CHOLERATOXIN AND ADRENERGIC COMPOUNDS (Fig. 4). Cholera toxin and β -adrenergic agents have been characterized pharmacologically as effective stimulators of adenylate cyclase in nearly every tissue tested, including lymphocytes (14, 15, 17, 20). Both are inducers in both our assays. Cholera toxin gave maximal induction of Thy-1⁺ cells at a concentration of 1 $\mu\text{g/ml}$ (panel 1) and of CR⁺ cells at 0.01 $\mu\text{g/ml}$ (panel 2). In both assays induction by cholera toxin was prevented by imidazole (10^{-7} M) added 10 min before the inducer, whereas theophylline (10^{-3} M) enhanced induction by suboptimal concentrations of cholera toxin.

The adrenergic agent epinephrine (panels 3 and 4) gave maximal induction of both Thy-1⁺ and CR⁺ cells at a concentration of 10^{-4} M. Both inductions were inhibited by incubation (for 10 min) with propranolol (10^{-5} M) before the addition of epinephrine; whereas in both assays phentolamine (an α -adrenergic blocking agent), at the same concentration and under the same conditions, enhanced induction by suboptimal concentrations of epinephrine, and reduced induction by optimal concentrations of epinephrine. This suggests that the β -receptor, but not the α -receptor, serves for induction in both assays, and that

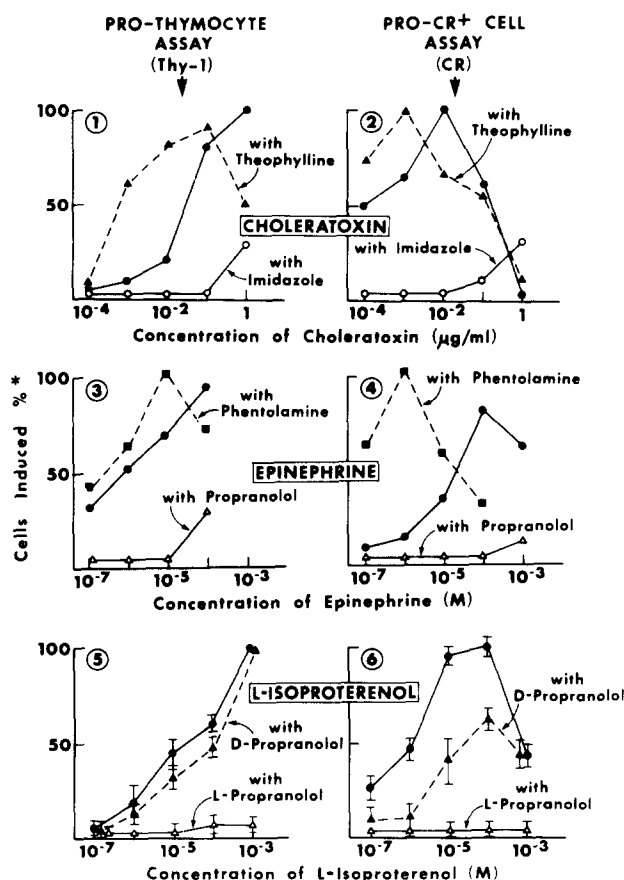


FIG. 4. Induction by three activators of adenylate cyclase: cholera toxin, epinephrine, and L-isoproterenol. The pharmacological data shown conform to the interpretation that activation of adenylate cyclase leads to induction regardless of which adenylate-cyclase-linked receptor is engaged. Thus induction by cholera toxin was inhibited by imidazole (10^{-7} M) and enhanced by theophylline (10^{-3} M) (panels 1 and 2). Induction by epinephrine was inhibited by propranolol (10^{-5} M) and enhanced by phentolamine (10^{-5} M) (panels 3 and 4). Induction of L-isoproterenol was inhibited by L-propranolol (10^{-5} M) rather than by D-propranolol (10^{-5} M) (panels 5 and 6). *Data standardized as percent maximal induction (see footnote to Fig. 2). (Mean values for three to four tests.)

effects of epinephrine mediated by α -adrenergic receptors, known in other systems (18), antagonize induction.

L-isoproterenol, a selective β -adrenergic agonist (18, 19) was inductive in both assays (panels 5 and 6). L-propranolol (10^{-5} M) completely inhibited induction by L-isoproterenol, in concentrations up to 10^{-3} M, in both assays. By contrast, D-propranolol (10^{-5} M), the dextrorotatory d-(+) isomer, did not significantly affect prothymocyte induction by L-isoproterenol and only partially inhibited induction of pro-CR⁺ cells by L-isoproterenol. This suggests that under the conditions of our assays the observed effect of propranolol is predominantly receptor-specific and is not due to non-specific membrane effects or to PDE activation (18).

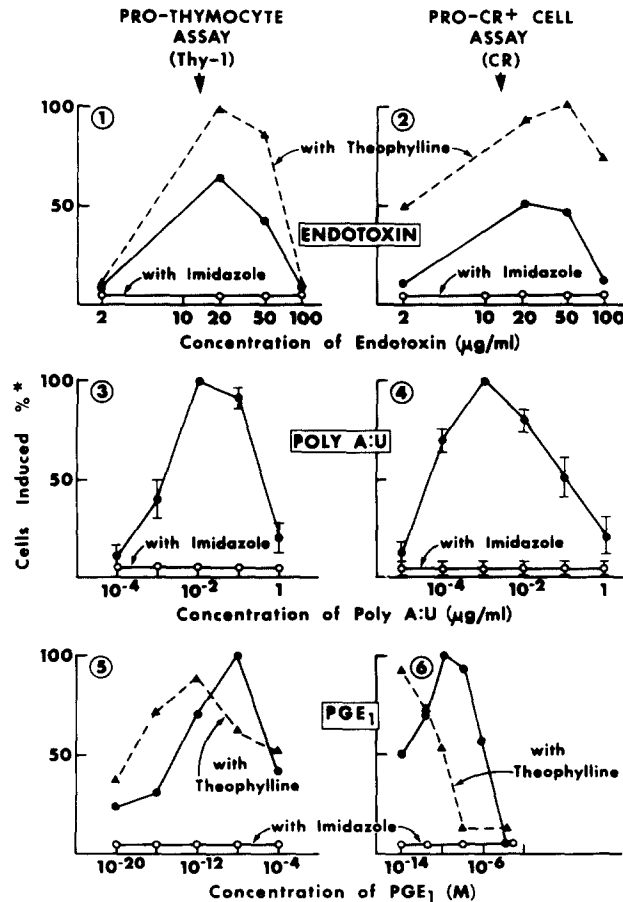


FIG. 5. Induction by endotoxin, poly A:U and PGE₁. The pharmacological data shown conform to the interpretation that induction by these three agents also involves a cAMP-dependent mechanism. (Concentrations of imidazole and theophylline as in Fig. 4). * Data standardized as percent maximal induction (see footnote to Fig. 2). (Mean values for four to five tests.)

(d) TESTS WITH OTHER INDUCING AGENTS (Fig. 5). Endotoxin (panels 1 and 2) caused induction in both assays, maximal at 20 $\mu\text{g/ml}$ and less at higher concentrations; concentrations above 100 $\mu\text{g/ml}$ were toxic). Induction was fully inhibited by imidazole (10^{-7} M), and was enhanced by theophylline (10^{-3} M). Lipid A gave similar results. Poly A:U (panels 3 and 4) gave optimal induction at a concentration of 10^{-2} - 10^{-3} $\mu\text{g/ml}$, both inductions being fully inhibited by imidazole (10^{-7} M).

Prostaglandin PGE₁ (panels 5 and 6), which is linked to adenylate cyclase activation in various systems (18), including mouse lymphocytes (20), gave maximal induction of prothymocytes at a concentration of 10^{-8} M and of pro-CR⁺ B cells at 10^{-10} M. Induction was inhibited by imidazole (10^{-7} M). Theophylline (10^{-3} M) enhanced induction by suboptimal concentrations of PGE₁, and inhibited induction by optimal or higher concentrations.

VII. Features of Late B-Cell Differentiation: The PC⁺ Phenotype. PC is the

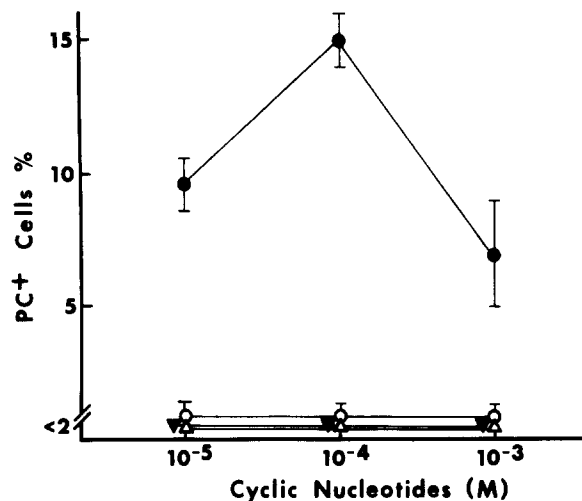


FIG. 6. Cyclic nucleotides: dose-response curves for induction of PC expression by cAMP (DBcAMP) and cGMP (8-bromo-cGMP) on Peyer's patch cells. Note: (a) induction by cGMP at lower concentrations is inhibited at higher concentrations, and (b) there is no induction by cAMP throughout the range of concentrations tested. (Mean values of four tests.) For B6-PC⁺: cGMP ●, cAMP ○; for B6 cells (PC⁻ congenic control): cGMP ▲, cAMP △.

last of the known surface markers expressed during sequential B-cell differentiation (6). The penultimate B-cell population, required for assaying PC induction, is Ig⁺:CR⁺:PC⁻. (We find that this population contains cells that are not immediately PC-inducible, presumably either because they have not completed an intermediate differentiative step or because they belong to a subset committed to a different terminal program.) The inducible population for the PC system was Peyer's patch cells prepared from mice treated with *C. parvum*.

(A) CYCLIC NUCLEOTIDES. In contrast to early T- and B-cell induction (Section VI, above), the terminal phenotype PC was induced by cGMP, not cAMP. The dose-response relations (Fig. 6) show inhibition of induction with high concentrations of cGMP, as was the case with cAMP in early T- and B-cell inductions (Fig. 3). Similarly cAMP inhibited induction of PC by cGMP, as cGMP inhibited cAMP-mediated early T- and B-cell induction (Fig. 3).

(B) PHARMACOLOGY. If PC-induction is mediated by cGMP, the acetylcholine analog carbachol should act in this system as an inducer rather than an inhibitor (which it is in early T- and B-cell induction assays [(Fig. 3, panels 3 and 4)]. Fig. 7 indicates that this is so.

(C) THYMOPOIETIN. The particular importance of thymopoietin is its claim to be a selective inducer of T-cell differentiation in the dual assay (see above, and reference 8). We therefore paid much attention to its action in the PC induction assay, especially because we had seen that thymopoietin partially inhibits CR⁺ B-cell induction by cAMP or ubiquitin (data not shown), and totally inhibits induction of Ia (21). As Fig. 7 shows, a concentration of thymopoietin that is optimal for T-cell induction (Fig. 2) completely inhibited induction of PC by carbachol. A possible explanation is that thymopoietin is an inducer of PC, and that its ostensible suppression of induction by carbachol might be attributed to high-dose inhibition (thymopoietin plus carbachol) which

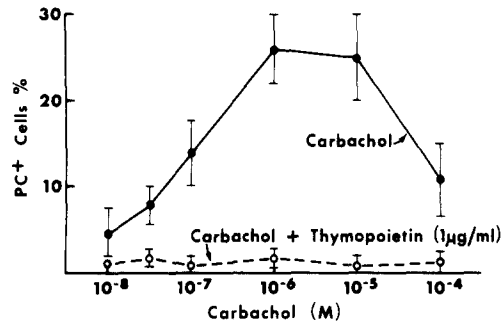


FIG. 7. Effect of thymopoietin (1 $\mu\text{g/ml}$) on induction of PC expression by carbachol. Note: thymopoietin inhibits induction by carbachol over the entire dose-response range. (Mean values of five tests.)

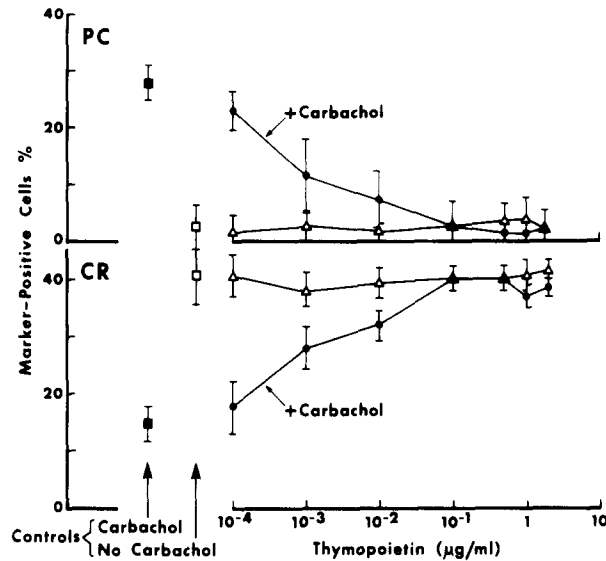


FIG. 8. Dose-response data for the effects of thymopoietin on carbachol-induced PC expression (upper panel), and on carbachol-induced reduction of CR expression (lower panel). These data illustrate that thymopoietin neither induced the expression of PC nor diminished the expression of CR, but suppressed both these effects of carbachol. (Mean values of three to eight tests.)

is documented for other inducers (Figs. 4 and 5). But this is not so, because thymopoietin should then enhance PC induction by a suboptimal concentration of carbachol, which it does not (Fig. 7). We confirmed this by assaying thymopoietin, over a wide range of concentrations, both alone and in combination with an optimal concentration of carbachol (10^{-6} M), for its effects on induction of PC expression. The upper panel of Fig. 8 shows that thymopoietin did not induce PC expression at any concentration tested, and that at no point did thymopoietin have any effect other than inhibition of PC induction by carbachol. The lower panel of Fig. 8, which refers to concomitant assays for CR expression with the same Peyer's patch cell suspension, shows that thymopoietin alone had no effect on the expression of CR, but that it prevented the fall in

proportion of CR⁺ cells that is characteristic of carbachol. We think it likely that PC expression and CR reduction are inversely correlated phenotypic changes affecting one cell set as it differentiates (though this wants proof). But regardless of mechanism these results signify that receptors for thymopoietin are present on Ig⁺:CR⁺:PC⁻ cells.

Discussion

We judged CR, scored in the presence of EDTA, to be the most unequivocal routine marker for early B cells, and PC the obvious choice for late B cells. For T cells, Thy-1 is the best routine early marker. There is no well-authenticated late immunogenetic marker, comparable to PC, for T cells. But there are useful data on regulation of T-cell functions, to represent late phases of T-cell differentiation (20, 22, 23; reviewed in 24, 25), analogous to data for B-cell functions (26).

The principle of the dual assay, in which the induction of two different cell populations is observed simultaneously, has proved invaluable. It has allowed an explanation of why an assortment of agents, many seeming physiologically incongruous and with varied pharmacological actions, can induce prothymocytes *in vitro*. It is now amply substantiated that prothymocytes and homologous cell sets are preprogrammed for particular phenotypes, and that only thymopoietin, among the variety of prothymocyte-inducing agents tested, acts selectively to induce this particular committed cell set.

The application of dual assays in other induction systems now reveals that thymopoietin probably has a more comprehensive role in lymphocyte regulation than was at first supposed. Thymopoietin's selective inductive action might have been explained by restriction of thymopoietin receptors to the prothymocyte. Evidently this is not so. The ability of thymopoietin to inhibit induction of PC (and Ia [21]) completely, and of CR partially (data not included), is no less striking than its selective induction of prothymocytes. Thus thymopoietin receptors are evidently present on B cells also, though presumably geared in this case to a biochemical circuit that inhibits rather than initiates induction. Ubiquitin is a nonselective inducer which evidently engages a different receptor, doubtless with its own biochemical linkage, which accords with other experiments in which we have found that ubiquitin can override the inhibition of CR induction produced by thymopoietin.

Can all these facts relating to initiation and inhibition of induction be plausibly related to a general scheme of differential regulation by intracellular cyclic nucleotides?

First, induction of Thy-1 and of CR can be viewed as early steps in the differentiation of T and B cells, respectively, and both are evidently linked to cAMP. Secondly, induction of T-cell functions (24, 25), and induction of PC (and of B-cell functions; reviewed in 26), can be viewed as late steps in the differentiation of T and B cells, respectively, and these evidently are linked to cGMP. This conforms to a scheme in which the exogenous cyclic nucleotides (which are inducers in their own right), and similarly the non-specific agents which affect levels of intracellular cyclic nucleotides, have contrasting effects on the early and late differentiative steps referred to, and make no distinction between T cells and B cells. Thymopoietin on the other hand has opposite effects on T and

B-cell differentiative steps, both early and late.²

We do not infer that the nonselective induction observed in dual assays *in vitro* has no physiological counterpart *in vivo*. On the contrary, this may be a pointer to regulators which are necessary to govern lymphocyte populations as a whole *in vivo*. Possibly the serum factor *facteur thymique serique* (27, 28), which has properties similar to ubiquitin *in vitro*, including sensitivity to inhibition by adrenergic blocking agents (29), could qualify for such a role. Thymopoietin, in this frame of reference, plays the part of a differential regulator of lymphocyte sub-populations.

Differential regulation of lymphocyte sub-populations by thymopoietin can be considered in terms that are familiar in the context of hormone-receptor interactions (Sonenberg and Schneider; 30) and which include—(a) degradation of the hormone into inductive and other fragments, at the plasma membrane or in the cytoplasm, (b) alternative modes of coupling of the receptor to one or another effector mechanism in the plasma membrane, and (c) ensuing activation of particular effector mechanisms and intervention of second messengers.

There are good precedents, especially in neurotransmission, for the production of contrasting effects on cellular cAMP and cGMP by the same agent acting on cells of different sets (31, 32). Ontogeny in the lymphoid system (and doubtless generally) requires the balanced output and inter-regulation of multiple cell sets that emanate from a common stem set and that become separately but coordinately programmed for cooperative functions. Thymopoietin, with its reciprocal effects on facets of T and B lymphocyte differentiation that depend on cyclic nucleotides, appears well fitted for the role of differential regulator, and this affords the most plausible explanation for the inclusion of thymopoietin receptors in the phenotypes of different classes of lymphocyte.

Summary

Results with a dual assay, for the induction of Thy-1⁺ T cells and of CR⁺ B cells from marker-negative precursors, confirm that thymopoietin is at present the only known selective inducer of prothymocytes. In contrast, various inducers, including ubiquitin, are active in both assays. Pharmacological evidence indicates that there are different cellular receptors for ubiquitin and thymopoietin.

Prothymocytes and pro-CR⁺ B cells compose two distinct populations in bone marrow and spleen; their distribution in density gradients is different, and elimination of either population enriches the other proportionately.

² Measurements of cellular cAMP in the standard cell population used for induction have shown significant elevation with nonselective inducers (poly A:U, endotoxin, ubiquitin) but not so far with thymopoietin (R. G. Coffey, M. P. Scheid, and J. W. Hadden, unpublished observations). The reason for this may be purely technical, reflecting the smaller number of cells induced by thymopoietin. Because only limited enrichment of the prothymocyte population is as yet feasible, elevation of cAMP induced selectively in prothymocytes by thymopoietin may be too small to measure under present conditions of testing. We are investigating the use of α -Lyb-2 sera to eliminate B cells (in which we assume cAMP to be elevated by the nonselective inducers) and thus achieve adequate enrichment for prothymocytes. Indications that thymopoietin elevates cGMP in cell populations rich in mature peripheral T cells (23) conform to the argument developed further in this discussion.

There are no noteworthy differences between induction of these two populations in regard to (a) kinetics, (b) dependence on temperature and protein synthesis, (c) activation by cAMP, and (d) inhibition by cGMP. The opposite inductive effects of cAMP and cGMP were corroborated by the use of pharmacological agents that raise or lower the levels of intracellular cyclic nucleotides.

In contrast, a third induction assay, which monitors acquisition of the PC⁺ surface phenotype, indicates that this differentiative step, the last known for B cells, is initiated by cGMP and inhibited by cAMP.

Induction of PC is also inhibited by thymopoietin, signifying that the inductive selectivity of thymopoietin is not due to restriction of its receptors to the T lineage cells. Rather it seems that receptors for thymopoietin occur also on PC-inducible and other B cells, although in this case geared biochemically to inhibition rather than expression of the succeeding gene program. This suggests a role for thymopoietin in the coordinated interregulation of lymphocyte classes, in addition to its better-known function as the thymic inducer of prothymocytes.

Present data conform to a general scheme in which the cyclic nucleotides cAMP and cGMP, and agents that affect intracellular levels of these mediators, influence reciprocally the early and late (functional) phases of lymphocyte differentiation as a whole, while thymopoietin influences reciprocally the differentiation of the B and T classes of lymphocyte.

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