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Induction of a T-cell specific antigen on bone marrow lymphocytes with thymus RNA

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Summary. Expression of a rabbit T-cell specific antigen can be induced on bone marrow lymphocytes following exposure to an RNA extract obtained from the thymuses of young rabbits. The presence of the antigen was demonstrated using goat anti-rabbit T-cell serum in a complement-dependent cytotoxicity assay. The T-cell antigen first appeared 3 h after addition of the thymus RNA to bone marrow cell cultures and the maximum number of cells expressing the T-cell antigen was observed within 24 h. RNA obtained from a source other than the thymus was found to be ineffective in inducing expression of the T-cell antigen. The induction of the antigen appears to be dependent on the presence of intact thymus RNA, as RNase treatment, but not trypsin treatment, destroyed the ability of the RNA to induce the T-cell antigen.

INTRODUCTION

Numerous attempts have been made to induce the formation of T cells from bone marrow lymphocytes or circulating null lymphocytes. One of the most successful methods has been with thymosin, a thymic hormone extracted from calf thymus (Goldstein, Asanuma, Battisto, Hardy, Quint & White, 1970; Bach, Dardenne, Goldstein, Guha & White, 1971; Goldstein, Guha, Katz, Hardy & White, 1972). Thymosin is a family of heat-stable acidic polypeptides (Goldstein, Low, McAdoo McClure, Thurman, Rossio, Lai, Chang, Wang, Harvey, Ramel & Meienhofer, 1977) capable of inducing the expression of various T-cell characteristics, and of converting functionally immature T cells into immunologically mature T cells (Cohen, Hooper & Goldstein, 1975). Another thymus hormone, also extracted from bovine thymus, which induces T cell maturation is thymopoietin (Basch & Goldstein, 1974; Goldstein, 1975; Basch & Goldstein, 1975). Concentrations of this polypeptide hormone, less than 1 nanogram, have been reported to induce the expression of T-cell markers on precursors present in the bone marrow. In addition, thymus humoral factor (THF) (Rotter, Schlesinger, Kalderon & Trainin, 1976), lymphocyte stimulating hormone (LSH) (Robey, 1975), and other thymic extracts (Friedman, 1975) have been reported to induce the formation of various T-cell characteristics.

The mode of action of these thymic preparations is unknown. However, the induction of T-cell characteristics and functions is apparently dependent upon the presence of existing T-cell precursors. If such precursors have not been produced, as is apparently the case in severe combined immunodeficiencies (Incefy, Boumsell, Touraine, L'Esperance, Smithwick, O'Reilly & Good, 1975), the thymic polypeptide hormones will be ineffective in triggering T-cell maturation.

In the course of a study aimed at learning more

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about T-cell maturation in the rabbit, it was found that expression of a T-cell specific antigen could be induced on bone marrow cells by RNA extracted from the thymus. Also, induction of the T-cell antigen, which appears on bone marrow cells within 3 h after exposure to thymus RNA, appears to be dependent on the presence of undegraded thymic RNA. This finding may provide additional insight into the mechanism of action of thymic hormones and possibly an avenue for stimulating T-cell maturation in conditions refractory to thymic hormone treatment.

MATERIALS AND METHODS

Preparation of cell suspensions

White domestic rabbits, 4–6 months of age, were killed by exsanguination and the thymus and spleen promptly removed and placed in cold Hanks's balanced salt solution (HBSS). The long bones from all limbs were removed and placed on crushed ice.

Thymocyte and spleen cell suspensions were prepared from thoroughly washed organs from which adherent fat and blood vessels had been removed. The tissue was gently minced in HBSS with mincing forceps to release cells from the connective tissue. Cell aggregates were disrupted by gentle aspiration using a needle and syringe. The cell suspension was then filtered through several thicknesses of gauze, collected by centrifugation, and washed three times in HBSS.

Bone marrow cells were obtained by first removing all flesh from the femur, tibia, and humerus. The bones were broken with strong pincers and cells were flushed from the marrow cavity into a petri dish with HBSS under gentle pressure using a Pasteur pipet. Cell suspensions were obtained by repeated gentle aspiration of the tissue through the orifice of a 5 ml plastic syringe and clumps of cells were removed by filtration through several layers of sterile gauze. The suspended bone marrow cells were collected by centrifugation and washed three times in HBSS.

Cells were counted using a haemocytometer and their viability determined by the trypan blue exclusion method.

Purification of bone marrow lymphocytes

Purified bone marrow lymphocytes were obtained by isopycnic centrifugation using Ficoll-paque (Pharmacia Fine Chemicals). Packed bone marrow cells were diluted 1 : 5 (v/v) in HBSS and 3 ml of the bone marrow cells were carefully layered on 3 ml of Ficoll-paque contained in conical glass centrifuge tubes. After centrifugation for 30 min at 400 *g*, the lymphocyte layer was carefully removed and washed twice with HBSS.

Preparation of anti-T-cell serum

Goat anti-rabbit T-cell serum (ATS) was prepared by a procedure similar to that described by Fradelizi, Chou, Cinader & Dubiski (1973). Briefly, from a rabbit thymocyte suspension, an injection dose containing 10^9 cells in 5 ml of HBSS was prepared. Half of the total dose was injected intravenously and half intramuscularly into an adult female goat. Injections of this type were given every 2 weeks for 10 weeks. Serum used in this study was obtained 2 weeks following the last injection.

To render the antiserum specific for rabbit T cells, the antiserum was heat inactivated at 56° for 30 min, absorbed 6 times for 30 min at 37° with 0·2 vol of packed rabbit erythrocytes, and 5 times with 0·2 vol of packed rabbit bone marrow cells.

Extraction of RNA

RNA was extracted by a hot phenol extraction procedure similar to that described by Scherrer & Darnell (1962). Briefly, 4–5 months old rabbits were killed by exsanguination and the thymuses were aseptically removed. Blood vessels and fat tissue were carefully removed and the tissue was washed in cold HBSS. The thymus tissue was then homogenized at 4° for 3 min using a Virtis homogenizer in a freshly distilled phenol solution saturated with Buffer A (0·5% sodium dodecyl sulphate, 10 mM sodium acetate, pH 5·1, 8 μg/ml polyvinyl sulphate, and 0·1% 8-hydroxyquinoline). After homogenization, an equal volume of Buffer A containing 1 mg/ml of washed bentonite was added to the homogenate and homogenization was continued for 3 min. The mixture was then heated to 55° with vigorous stirring and then rapidly cooled in an ice-water bath. When the temperature of the solution was below 6° the phenol and aqueous phases were separated by centrifugation at 10,000 *g* for 10 min at 4°.

Following centrifugation the aqueous phase was carefully removed and mixed with an equal volume of buffer-saturated phenol. This mixture was heated

to 55° with stirring, cooled, and the phases separated by centrifugation. This extraction procedure was repeated 3–4 times until the interface between the phenol and aqueous phase was virtually free of precipitated protein.

Three volumes of cold 95% ethanol was added to the final aqueous phase and the RNA precipitated overnight at −20°. After removing visible DNA in the precipitate with a glass rod, the precipitated RNA was collected by centrifugation at 20,000 *g* for 15 min at 4°. The precipitate was dissolved in saline containing 5 mM magnesium chloride, electrophoretically purified deoxyribonuclease I (Worthington Biochemical Corporation) was added to a final concentration of 20 μg/ml, and the solution was incubated at room temperature for 30 min. The DNase was then removed by 2 phenol extractions as described above. Again 3 volumes of cold ethanol were added to the final aqueous phase and the RNA precipitated overnight at −20°.

Following the additional ethanol precipitations, the precipitated RNA was collected by centrifugation, dissolved in saline, and the RNA concentration determined. This was done spectrophotometrically using 20 OD_{260} units as equivalent to 1 mg of RNA. The RNA was then divided into aliquots, each containing a known amount of RNA, and stored in ethanol at −20° until needed. All RNA extracts used in this work had an $OD_{260/280}$ ratio of 1·9 to 2·0, indicating that the preparations were relatively free of protein.

Following preparation of a bone marrow cell suspension, bone marrow cell RNA was obtained in exactly the same manner as used for extracting thymus RNA.

Sucrose density gradient centrifugation

To determine if degradation of the RNA had occurred during extraction, portions of all RNA extracts were subjected to sucrose density gradient centrifugation. The precipitated RNA was dissolved in 0·1 M sodium chloride containing 1 mM magnesium chloride. Then, 0·1–0·2 ml containing 100–150 μg of RNA was carefully layered on a 5 ml linear gradient composed of 5–30% sucrose. The gradient was then centrifuged for 3 h at 140,000 *g* using a Beckman SW 50·1 swinging bucket rotor in a Sorvall OTD-II ultracentrifuge. Following centrifugation, fractions were collected from the bottom of the tube and the concentration of RNA in each fraction measured spectrophotometrically.

Induction of T-cell antigen

To induce the expression of a T-cell antigen on bone marrow cells, precipitated RNA was first collected by centrifugation at 20,000 *g* for 10 min at 4°. After all ethanol had been removed by desiccation, the RNA was then dissolved in RPMI-1640 culture media. Next, 0·2 ml of the RNA extract containing varying known amount of RNA was added to 10^7 bone marrow cells contained in 0·2 ml of RPMI-1640 culture media.

Detection of a T-cell specific antigen on bone marrow cells

Following incubation at 37° in a CO_2 incubator for various periods of time, the bone marrow cells which had been incubated with RNA were washed twice and suspended in HBSS to a concentration of 10^6 cells/ml. To detect cells expressing a T-cell specific antigen, a complement dependent cytotoxicity assay as described by Fradelizi *et al.* (1973) was performed using a 1 : 4 dilution of ATS. This dilution of ATS continually demonstrated greater than 95% cytotoxicity for rabbit thymocytes, 50–55% cytotoxicity for rabbit spleen cells, and insignificant (background) cytotoxicity for bone marrow cells. Also, to insure objectivity, cell cultures were coded so that the individual doing the cytotoxicity assay was unaware of the treatment the various cell cultures had received.

Cytotoxicity index

To evaluate the sensitivity of the RNA-treated bone marrow cells to ATS, a cytotoxicity index (Isaković, Mitrović, Marković, Rajčević & Janković, 1975) was determined for each culture. The calculation used for determining the cytotoxicity index, which is expressed as a percent, is:

$$\text{CI}\ (\%) = \frac{\text{Dead cells in test} - \text{Dead cells in control (mean)}}{\text{Viable cells in control (mean)}} \times 100$$

For control cultures, pre-immune serum was used instead of ATS in the complement dependent cytotoxicity assays.

Enzymatic treatment of RNA

In some experiments, the thymus RNA extracts were treated with either ribonuclease (RNase) (Worthington Biochemical Corporation) or trypsin (Worthington Biochemical Corporation) prior to addition of bone marrow cells. For this, 20 μg of

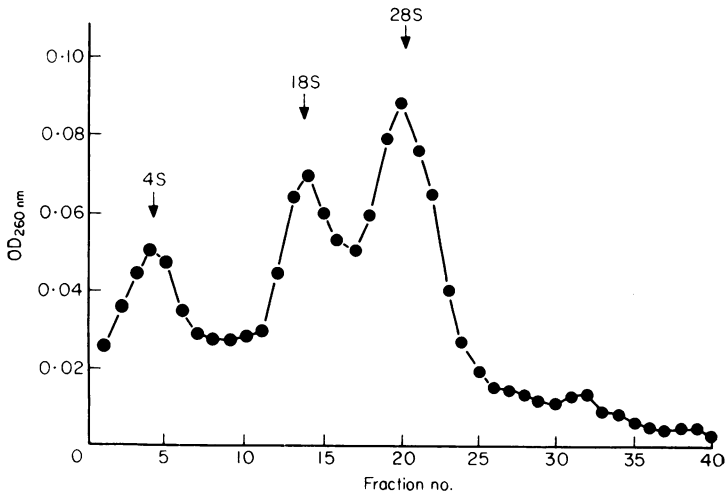

Figure 1. Sucrose density gradient profile of thymus RNA.

RNase was added to 500 μg of RNA (enzyme substrate ratio of 1 : 25) and the mixture incubated at 37° for 30 min. Also, 5 μg of trypsin was added to 500 μg of RNA (enzyme substrate ratio of 1 : 10) and the mixture incubated for 30 min at 37° for 30 min.

RESULTS

Sucrose density gradient centrifugation of RNA

A typical sucrose density gradient profile of the extracted thymus RNA can be seen in Fig. 1. In all RNA preparations used, the 18S and 28S peaks were higher than the 4S peak, indicating little degradation of the RNA occurred during extraction.

Induction of a T-cell antigen on unfractionated bone marrow cells

Various concentrations of thymus RNA were mixed with a constant number of unfractionated rabbit bone marrow cells. After incubation for various periods of time, the cells were collected by centrifugation, washed twice, and the cytotoxicity index determined for each culture. As shown in Table 1, unfractionated bone marrow cells incubated with 250–750 μg of RNA demonstrated a significant

Table 1. Induction of a T-lymphocyte specific antigen on unfractionated bone marrow cells by thymus RNA

Amount of RNA added (μg)	Cytotoxicity index (%) after culturing*				
	0 h	3 h	12 h	24 h	48 h
None	0·9 (±2·1)	1·3 (±3·3)	1·9 (±2·5)	1·9 (±1·9)	1·3 (±6·0)
250	0·3 (±2·9)	5·3 (±1·9)	6·6 (±4·3)	8·2 (±2·8)	9·4 (±2·8)
500	1·2 (±2·4)	8·3 (±4·3)	10·9 (±6·6)	15·5 (±2·9)	19·4 (±5·7)
750	1·1 (±2·1)	7·5 (±2·2)	9·2 (±2·4)	13·8 (±4·3)	17·6 (±3·7)

* Each value is the mean (±s.d.) obtained from six identical experiments.

Table 2. Induction of a T-lymphocyte specific antigen on purified bone marrow lymphocytes by thymus RNA

Source of RNA	Cytotoxicity index (%) after culturing*				
	0 h	3 h	12 h	24 h	48 h
Thymus (500 μg)	0·55 (±1·6)	24·3 (±6·7)	38·3 (±6·1)	41·9 (±7·5)	34·6 (±6·2)
Bone marrow (500 μg)	0·6 (±1·2)	2·3 (±1·8)	5·4 (±2·6)	6·9 (±3·4)	7·6 (±3·8)
No RNA added	0·5 (±1·6)	1·6 (±1·6)	1·9 (±1·4)	1·0 (±4·4)	1·43 (±2·6)

* Each value is the mean (±s.d.) obtained from six identical experiments.

increase (as determined by the F test, with $\alpha = 0{\cdot}005$) (Chao, 1969) in sensitivity to ATS, as compared to control cultures which contained no RNA. An initial increase in the sensitivity to ATS was noted after only 3 h of incubation and continued to 48 h. Also, data presented in Table 1 indicates that 500 μg of thymus RNA is optimal for inducing a T-cell antigen for the conditions of the experiment.

Induction of a T-cell antigen on purified bone marrow lymphocytes

Rabbit bone marrow normally contains a number of different cell types in various stages of development, including approximately 22% lymphocytes (mature and immature), as determined in our laboratory. As we were concerned with a phenomena apparently involving lymphocytes, a clearer indication of the ability of thymus RNA to induce expression of a T-cell antigen would be possible if only bone marrow lymphocytes were used in the study. Therefore, purified (>90%) bone marrow lymphocytes were obtained using isopycnic centrifugation as described above.

To ascertain the ability of thymic RNA to induce expression of a T-cell antigen on purified bone marrow lymphocytes, a constant amount of RNA, 500 μg, was incubated with 10^7 lymphocytes. After incubation for various periods of time the sensitivity of the cells to ATS was determined and a cytotoxicity index calculated for all cultures. As can be seen in Table 2, a substantial increase in the cytotoxicity index occurred following exposure of the purified bone marrow lymphocytes to thymus RNA.

Also, the ability of thymus RNA was compared with RNA extracted from bone marrow cells to induce expression of a T-cell antigen on bone marrow lymphocytes. As shown in Table 2, RNA extracted

Table 3. Effect of trypsin and RNase treatment on the induction of a T-lymphocyte specific antigen by thymus RNA

Treatment of thymus RNA	Cytotoxicity index (%) after culturing*				
	0 h	3 h	12 h	24 h	48 h
No enzyme treatment	0·5 (±1·1)	25·9 (±5·1)	31·4 (±5·2)	38·4 (±7·3)	37·4 (±7·3)
Trypsin treated	0·9 (±1·4)	20·5 (±3·1)	25·28 (±4·0)	29·7 (±6·2)	28·6 (±6·3)
RNase treated	1·1 (±1·7)	5·4 (±1·8)	3·6 (±2·6)	8·2 (±3·3)	8·5 (±2·9)
No RNA added to cell cultures	1·1 (±1·1)	1·9 (±1·6)	1·2 (±2·0)	2·2 (±1·6)	2·1 (±1·3)

* Each value is the mean (±s.d.) obtained from six identical experiments.

from bone marrow cells induced expression of a T-cell antigen on a small number of bone marrow lymphocytes. However, the number of bone marrow lymphocytes expressing a T-cell antigen following exposure to bone marrow RNA was only 16–19% of that noted following exposure to thymus RNA. These experiments demonstrate that exposure to RNA is not simply rendering the bone marrow lymphocytes nonspecifically more sensitive to ATS.

Effect of enzyme treatment on induction of a T-cell antigen

Finally, we wished to show whether or not RNA was the active molecular component involved in inducing expression of a T-cell antigen. To do this, thymus RNA was treated with either trypsin or RNase prior to incubation with bone marrow lymphocytes. As can be seen in Table 3, treatment with RNase reduced by approximately 80% the ability of the thymus RNA to induce expression of a T-cell antigen. However, trypsin treatment reduced the ability of the RNA to induce expression of the T-cell antigen by only 20–23%. This indicates that the active component involved in triggering expression of the T-cell antigen is probably RNA.

DISCUSSION

Interest in RNA extracted from lymphoid tissues has existed since Fishman's early observations that RNA extracted from macrophages which had been allowed to phagocytose bacteriophage could specifically transfer an immune response to lymphoid cells obtained from non-immunized animals (Fishman, 1961). Since then, transfer of immunity by RNA or RNA-antigen complexes has been reported by numerous investigators (Friedman, 1973).

RNA extracted from thymus tissue has also been shown to induce various immune phenomena. Cohen, Majer & Friedman (1969) have shown that RNA extracted from the thymuses of mice hyper-immunized with sheep erythrocytes could convert a small portion of peritoneal cells or spleen cells obtained from normal mice into antibody forming cells. Also, Nakamura (1976) has reported that a thymus RNA extract can induce the proliferation of plasma cells from bone marrow cells *in vitro*. The active RNA component in the extract had a 4S to 6S sedimentation value.

Our findings are unique in that thymus RNA obtained from non-immunized animals apparently triggers or accelerates T-cell maturation as indicated by the appearance of a T-cell specific antigen on some bone marrow cells. The marked difference in the sensitivity to ATS of cells incubated with thymus RNA and bone marrow RNA indicates that a T-cell specific antigen is being expressed on some cells rather than the RNA simply causing the cells to become nonspecifically more sensitive to ATS. If the cells were becoming nonspecifically more sensitive to ATS following incubation with RNA, the cytotoxicity index would be the same following incubation with either bone marrow RNA or thymus RNA. However, only a slight increase in the cytotoxicity index occurred when bone marrow cells were incubated with bone marrow RNA. This slight increase could indicate the degree to which the RNA rendered the cells nonspecifically more sensitive to ATS, or indicate actual expression of a T-cell antigen on a very small number of cells.

The molecular component involved in inducing expression of the T-cell antigen appears to be RNA, as expression of the T-cell antigen was reduced by about 80% following treatment of the extract with RNase. In contrast, only a 20% decrease in expression of the T-cell antigen occurred following treatment of the thymus RNA with trypsin. The small reduction observed following trypsin treatment of the thymus RNA could be due to contamination of the trypsin with RNase or possibly due to some detrimental effect of the enzyme on the bone marrow lymphocytes.

About 75% of the experiments in which we have attempted to induce expression of the T-cell antigen on bone marrow cells with thymus RNA have been successful. Also, we have always used the RNA preparations within 2 weeks following extraction and, therefore, do not know if the RNA remains active on long-term storage.

The mechanism by which thymus RNA induces the expression of the T-cell antigen on bone marrow cells has not been delineated. However, it is possible that the active component is messenger RNA that may either code for production of a T-cell antigen in bone marrow cells, or for a thymic maturation hormone, which, upon being incorporated into a cell, results in expression of this hormone and thus maturation of the pre T cells. Alternatively, the RNA may be involved in triggering the maturation process in a select group of cells by activating

expression of existing genetic information. Work is presently underway to determine the mechanism by which the thymus RNA induces expression of the T-cell antigen and whether or not other T-cell characteristics and T-cell functions are induced by exposure of bone marrow cells to thymus RNA.

This finding may, therefore, provide an opportunity to study not only the maturation of this important cell type, but perhaps also the mode of action of thymic hormones which also influence T-cell maturation.

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REFERENCES

BACH J.F., DARDENNE M., GOLDSTEIN A.L., GUHA A. & WHITE A. (1971) Appearance of T cell markers in bone marrow rosette forming cells after incubation with thymosin, a thymic hormone. *Proc. nat. Acad. Sci. (Wash.)*, **68**, 2734.

BASCH R.S. & GOLDSTEIN G. (1974) Induction of T-cell differentiation by thymin, a purified polypeptide hormone of the thymus. *Proc. nat. Acad. Sci. (Wash.)*, **71**, 1474.

BASCH R.S., & GOLDSTEIN G. (1975) Thymopoietin-induced acquisition of responsiveness to T cell mitogens. *Cell. Immunol.* **20**, 218.

CHAO L.L. (1969) *Statistics: Methods and Analyses*, p. 294. McGraw-Hill, New York and London.

COHEN E.P., MAJER J. & FRIEDMAN K. (1969) Responses to immunization in the thymus of the adult mouse. *J. exp. Med.* **130**, 1161.

COHEN G.H., HOOPER J.A. & GOLDSTEIN A.L. (1975) Thymosin-induced differentiation of murine thymocytes in allogeneic mixed lymphocyte cultures. *Ann. N.Y. Acad. Sci.* **249**, 145.

FISHMAN M. (1961) Antibody formation *in vitro*. *J. exp. Med.* **114**, 837.

FRADELIZI D.P., CHOU C.T., CINADER B. & DUBISKI S. (1973) A membrane antigen of rabbit thymus cells. *Cell. Immunol.* **7**, 484.

FRIEDMAN H. (1973) RNA in the immune response. *Ann. N.Y. Acad. Sci.* **207**, 5.

FRIEDMAN H. (1975) Thymus factors in immunity. *Ann. N.Y. Acad. Sci.* **249**, 5.

GOLDSTEIN A.L., ASANUMA Y., BATTISTO J.R., HARDY M.A., QUINT A. & WHITE A. (1970) Influence of thymosin on cell-mediated and humoral immune responses in normal and immunologically deficient mice. *J. Immunol.* **104**, 359.

GOLDSTEIN A.L., GUHA A., KATZ M.M., HARDY M.M. & WHITE A. (1972) Purification and biological activity of thymosin, a hormone of the thymus gland. *Proc. nat. Acad. Sci. (Wash.)*, **69**, 1800.

GOLDSTEIN A.L., LOW T.L.K., MCADOO M., MCCLURE J., THURMAN G.B., ROSSIO J., LAI C., CHANG D., WAND S., HARVEY C., RAMEL A.H. & HEIENHOFER J. (1977) Thymosin a_1: Isolation and sequence analysis of an immunologically active thymic polypeptide. *Proc. nat. Acad. Sci. (Wash.)*, **74**, 725.

GOLDSTEIN G. (1975) The isolation of thymopoietin (thymin). *Ann. N.Y. Acad. Sci.* **249**, 177.

INCEFY G.S., BOUMSELL L., TOURAINE J.L., L'ESPERANCE P., SMITHWICK E., O'REILLY R. & GOOD R.A. (1974) Enhancement of T-lymphocyte differentiation *in vitro* by thymic extracts after bone marrow transplantation in severe combined immunodeficiencies. *Clin. Immunol. Immunopath.* **4**, 258.

ISAKOVIĆ K., MITROVIĆ K., MARKOVIĆ B.M., RAJČEVIĆ M. & JANKOVIĆ B.D. (1975) Preparation of specific anti-thymocyte and anti-bursacyte sera in rabbits. *J. Immunol. Method.* **7**, 359.

NAKAMURA K. (1976) The proliferation of plasma cells from mouse bone marrow *in vitro*. II. Stimulation of IgG-producing cells by an RNase-sensitive thymocyte homogenate. *Cell. Immunol.* **25**, 163.

ROBEY W.G. (1975) Further characterization of an antibody-stimulating factor isolated from bovine thymus. *Ann. N.Y. Acad. Sci.* **249**, 211.

ROTTER V., SCHLESINGER M., KLADERON R. & TRAININ N. (1976) Response of human lymphocytes to PHA and Con A, dependent on and regulated by THF, a thymic hormone. *J. Immunol.* **117**, 1927.

SCHERRER K. & DARNELL J.E. (1962) Sedimentation characteristics of rapidly labelled RNA from Hela cells. *Biochem. biophys. Res. Commun.* **7**, 486.