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# Direct demonstration of immunoglobulin κ chain RNA in thymus T cells by *in situ* hybridization

(T and B cells/complementary DNA)

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ABSTRACT **Mouse thymuses with more than 99% T cells have been reported to contain immunoglobulin κ mRNA-like molecules (κ RNA) in relatively large quantities. The present study was undertaken to rule out the possibility that the κ RNA was mainly a product of a few contaminating B cells of the thymus and to determine whether all T-cell subpopulations contained κ RNA. By *in situ* hybridization with DNA complementary to κ mRNA (κ cDNA) the following observations were made: 98.5% of thymus cell preparations hybridized with κ cDNA; the 1.5% unlabeled cells were generally larger and paler staining than the majority of thymus cells. Only 0.015% of thymus cells were intensely labeled and appeared to be plasma cells. Also, 87% of spleen cells hybridized with κ cDNA; most of these showed similar labeling intensity to the majority of thymus cells. The number of unlabeled cells corresponded to the percentage of hemopoietic cells and macrophages in the spleen. Spleen cells in the range of 0.37–0.85% were intensely labeled and appeared to be plasma cells. The following controls supported the conclusion that the results with thymus and spleen were due to specific hybridization: most of the κ mRNA-deficient tissue culture cells of the plasmocytoid tumor ABPL-4 did not hybridize with κ cDNA. The κ mRNA-producing cells from myeloma PC 3741 hybridized *in situ* with κ cDNA. Furthermore, all cells from this tumor and all spleen cells hybridized uniformly with a cDNA probe complementary to most of the total cellular poly(A)-containing RNA species of these cells. These results indicate that T cells of all types in the thymus as well as in the periphery contain substantial quantities of κ RNA.**

It had previously been found by nucleic acid hybridization that mouse thymocytes contain a relatively large quantity of κ mRNA-like molecules (κ RNA) (1, 2,*). This was taken as evidence for κ RNA in T cells because over 99% of the thymus cells reacted with anti-theta serum in immunofluorescence tests. However, a doubt remained as to whether the small number of contaminating B cells might be extremely rich in κ RNA and totally account for the κ RNA levels found in the thymus. Furthermore, it was important to determine, if T cells were positive, whether or not each T cell subtype contained κ RNA.

In the present study these problems were assessed by *in situ* hybridization with [$^3$H]DNA complementary to κ mRNA (κ cDNA).

## MATERIALS AND METHODS

**Preparation of κ cDNA.** [5-$^3$H]dCTP-labeled DNA complementary to the c region and to the 3′ untranslated region of κ mRNA (κ cDNA) was prepared as described (2). Briefly, pure κ mRNA was prepared from membrane-bound ribosomes of MOPC-41 myeloma tumors by oligo(dT)-cellulose chromatography and polyacrylamide gel electrophoresis in formamide. The electrophoretically eluted translationally pure κ mRNA was transcribed into cDNA with avian myoblastosis virus reverse transcriptase. The κ cDNA was further purified by hybridization to pure κ mRNA. The κ cDNA was pure by the standards described (2). Its specific activity was approximately $10^7$ cpm/μg.

**Preparation of PU 5.1 "Whole Cell" cDNA.** Total RNA was prepared from the B lymphoid cell tumor PU 5.1, and the poly(A)-containing RNA was isolated by affinity chromatography on oligo(dT)-cellulose (3). Complementary DNA labeled with [5-$^3$H]dCTP was prepared from the poly(A)-containing RNA by the same method used for κ cDNA. This cDNA hybridized with its template over four orders of magnitude ($C_rt$ $\sim 5 \times 10^{-2}$ to $5 \times 10^2$) reaching 100% hybridization. It hybridized with whole cell poly(A)-containing RNA of EL 4 T cells and of mouse kidney to approximately 90% (unpublished data).

***In Situ* Hybridization.** *In situ* hybridization was a modification of the method of Harrison *et al.* (4, 5). Suspensions of BALB/c mouse spleen and thymus cells in Hanks' balanced salt solution containing 5% decomplemented fetal calf serum were prepared by gently teasing the tissues with two needles. This method releases only mobile cells, so that, for example, the thymus cell preparation is essentially free of epithelial cells and fibroblasts. ABPL-4 cells were spun out of tissue culture medium and suspended in salt solution/5% (vol/vol) fetal calf serum. PC 3741 myeloma cells which produce μκ immunoglobulins were grown in the ascites form in (BALB/c × NZB)$F_1$ mice. The cell suspensions were washed three times in salt solution/5% fetal calf serum and finally suspended in this solution. Glass slides and coverslips were cleaned in acid dichromate and kept in 95% (vol/vol) ethanol until use. For *in situ* hybridization a small drop of a cell suspension was placed on a cleaned slide and air dried. It was found that when smears were prepared RNA apparently leaked out of the cells so that hybridization occurred as a dark background of grains all over the slide. Air-dried slides were fixed in absolute methanol for 3 min at room temperature, treated with 0.2 M HCl at room temperature for 25 min, and dehydrated in 50%, 70%, and 90% ethanol. κ cDNA (2–10 μl) at 4800 cpm/μl in 40% (vol/vol) formamide 0.45 M NaCl/0.045 M Na citrate was placed over a premarked area on the slide and covered with a $5 \times 5$ mm$^2$ coverslip. The slides were incubated at 43° for 18 hr in a moist atmosphere. After hybridization the slides were washed in 0.3 M NaCl/0.03 M Na citrate at room temperature, kept for 1 hr at 55° in this solution, treated with S1 nuclease (Sigma) (600 units/ml) for 1 hr at 37°, and washed for 3 days in 0.3 M NaCl/0.03 M Na

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Abbreviations: κ RNA, RNA molecules that hybridize with DNA complementary to κ mRNA (κ cDNA); $C_rt$, concentration of RNA in hybridization reaction × the time of incubation, expressed as mol of nucleotide × sec/liter.

* Putnam, D., Storb, U. & Clagett, J. (1977) *Proceedings ICN-UCCA Meeting*, in press.

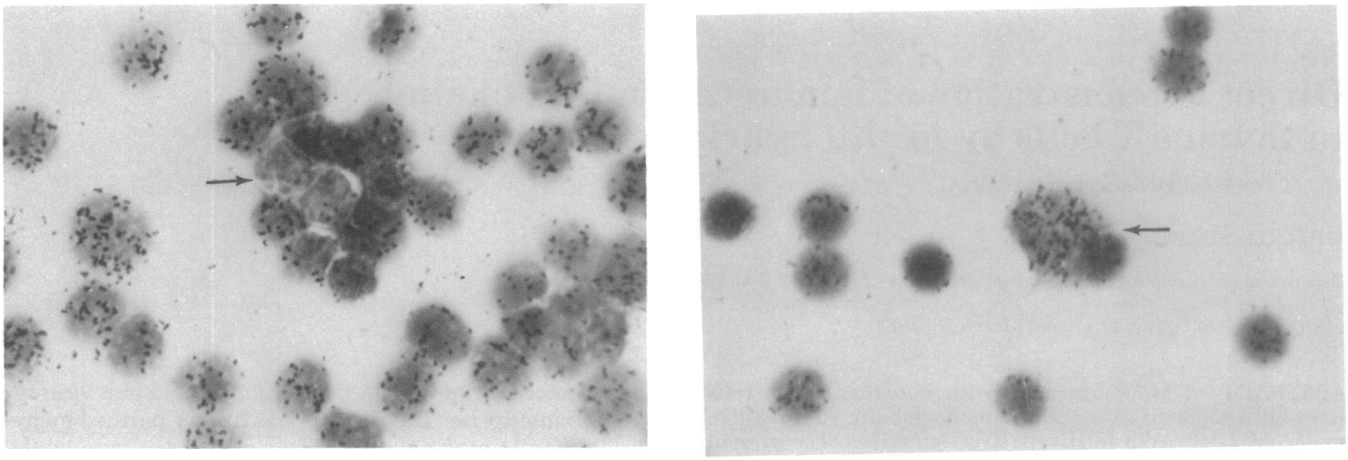


FIG. 1. *In situ* hybridization in 4-week old BALB/c thymus cells. The radioautographs were developed after 6 weeks of exposure subsequent to hybridization with 48,000 cpm of κ [$^3$H]cDNA. (*Left*) Most cells are labeled with 5 to 40 grains; cell indicated by arrow is negative. (*Right*) Cell indicated by arrow is probably a plasma cell.

citrate at 4°. Then radioautographs were prepared with Kodak NTB-2 emulsion. After 2–6 weeks incubation at 4°, the cell slides were developed and then stained with McNeal's stain.

## RESULTS

**Thymus.** After hybridization with 48,000 cpm of κ [$^3$H]-cDNA and 6 weeks of exposure, 98.5% of thymus cells were positive (Table 1 and Fig. 1 *left*). Only 1.5% of thymus cells were scored as negative (zero to four grains per cell). The negative cells were generally larger and stained paler than their neighbors on the slide. Their identity was not further analyzed. Only 8/53,400 thymus cells (0.015%) were too intensely labeled for their grains to be counted—one of these with the appearance of a plasma cell is shown in Fig. 1 *right*. In this context it is interesting that, when immunofluorescence with anti-mouse immunoglobulin serum is used, 0.02% of young BALB/c thymus cells appear to be plasma cells (D. Putnam and J. Clagett, personal communication). In another experiment, thymus cells were hybridized with only 24,000 cpm of κ [$^3$H]cDNA and the radioautographs were exposed for only 2 weeks. The majority of cells showed only zero to six grains (data not shown) and less than 0.02% of the cells were intensely labeled.

**Spleen.** After hybridization with κ cDNA and 6 weeks of exposure, 87% of spleen cells were positive. Most of these showed a labeling intensity similar to that of thymus cells. It was not possible to distinguish two classes of labeling intensity that might have corresponded to T and B cells (Table 1 and Fig. 2). Of the total spleen cells, 0.37% were intensely labeled. All of these were large and many of them had the appearance of plasma cells. The percentage of these heavily labeled cells is related roughly to the number of plasma cells found in normal mouse spleens (0.5%) (6). Furthermore, 13% of the spleen cells had not hybridized with κ cDNA. This value correlates with the numbers of hemopoietic cells and macrophages found in normal mouse spleens (6). These cells also provide a negative control for hybridization with κ cDNA, substantiating that the κ cDNA hybridization *in situ* is specific. Unfortunately, the cell morphology was badly preserved after *in situ* hybridization; therefore, although the numbers of highly positive and negative cells correlate with those of plasma cells and hemopoietic cells plus macrophages, respectively, their assignment remains indirect as yet.

In another experiment that screened for highly positive cells, spleen cells were hybridized with only 24,000 cpm of κ [$^3$H]-cDNA and the radioautographs were developed after 2 weeks; the majority of the cells had zero to six grains, as was found for the thymus. However, 0.85% of the cells were heavily labeled, most with the appearance of plasma cells (Fig. 3).

**Negative Controls.** A negative control for *in situ* hybridization with κ cDNA was provided by hybridization of κ cDNA with ABPL-4 tissue culture cells. These cells were derived from a κ RNA-producing plasmacytoid BALB/c mouse tumor. Upon prolonged passage in tissue culture, they showed drastically reduced levels of κ RNA—the average cell contained only 7 molecules of κ RNA (2) as compared to 1000 molecules before shutoff or to 100,000 molecules in myeloma cells. Most of these ABPL-4 cells appeared negative in *in situ* hybridization with κ cDNA (Fig. 4 *left*). Very few cells were slightly positive, mainly over the nucleus (Fig. 4 *right*).

Table 1. Distribution of grain counts after *in situ* hybridization with κ cDNA*

| | Distribution, % | | | |
|---|---|---|---|---|
| | 0–4 | 5–40 | >40 | Uncountable |
| Spleen | 13 | 86 | 1 | 0.37 |
| Thymus | 1.5 | 98.3 | 0.18 | 0.015 |

* The radioautographs were developed for 6 weeks and 48,000 cpm of κ [$^3$H]cDNA was used.

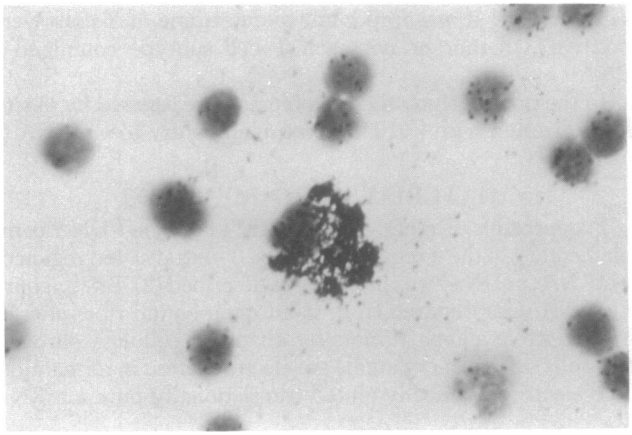


FIG. 2. *In situ* hybridization in 4-week old BALB/c spleen cells with 48,000 cpm of κ [$^3$H]cDNA. The radioautograph was developed after 6 weeks of exposure.

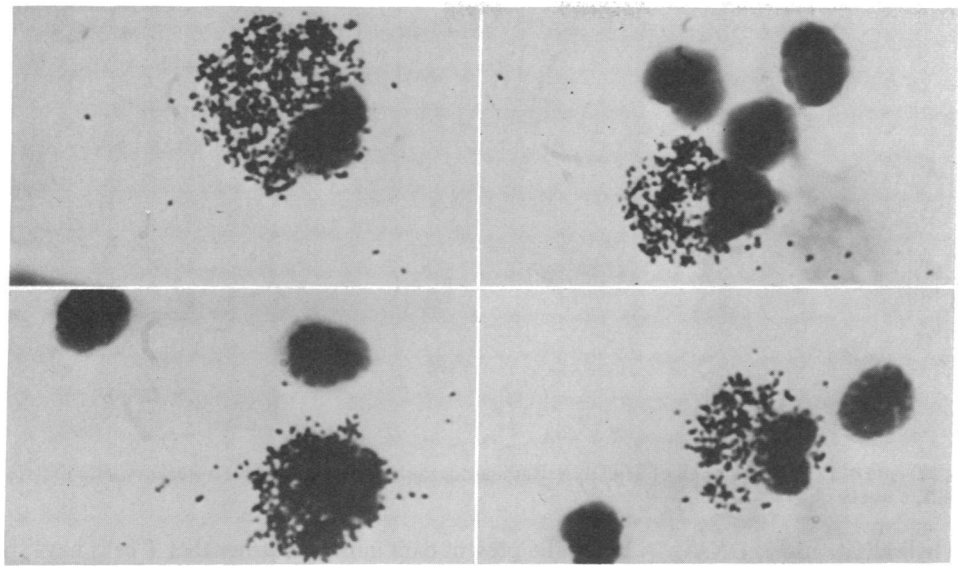
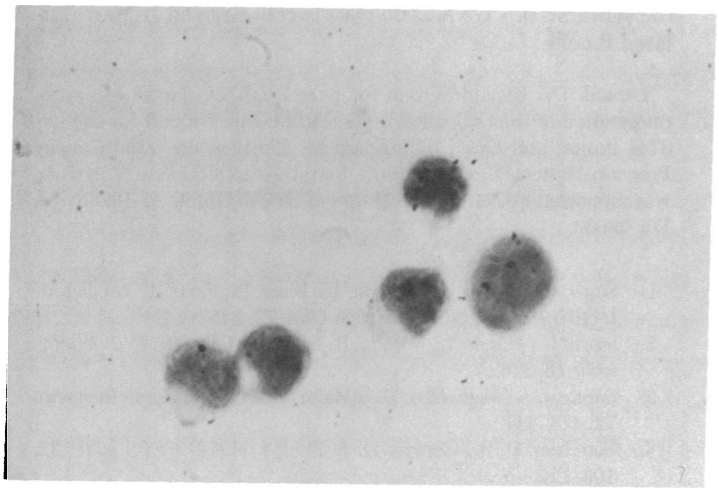


FIG. 3. *In situ* hybridization in 4-week old BALB/c spleen cells with 24,000 cpm *k* [$^3$H]cDNA. The radioautographs were developed after 2 weeks of exposure. Only 0.85% of cells were heavily labeled; four examples of such cells are shown

**Positive Controls.** (*i*) *With κ cDNA.* Myeloma PC 3741 cells which produce κ chains (6) were positive in every cell when hybridized *in situ* with a very small amount of κ cDNA (Fig. 5). The grain counts were lower than in the presumptive plasma cells of thymus and spleen (Figs. 1–3); this is probably mainly due to the smaller amount of κ cDNA used for *in situ* hybridization with the PC 3741 cells. (*ii*) *With "whole cell cDNA."* PU 5.1 whole cell cDNA was hybridized *in situ* with PC 3741 myeloma cells and spleen cells. In both cases, every cell was strongly positive with very little variation in labeling intensity between cells (Fig. 6).

## DISCUSSION

*In situ* hybridization with κ cDNA as used in this study appears to be specific for κ RNA-containing cells. First, the κ cDNA probe was pure by the criteria described (2). Briefly, the purified κ cDNA hybridized with purified κ mRNA to over 90% over a $C_rt$ range of about 100 with a $C_rt_{1/2}$ of $1.4 \times 10^{-3}$. A standard control of β-globin cDNA hybridized with its mRNA with a $C_rt_{1/2}$ of $7.5 \times 10^{-4}$. Because pure globin RNA can be relatively easily obtained and κ mRNA has about twice the complexity of β-globin mRNA, the comparison of the two $C_rt_{1/2}$ values and the plateau of hybridization reached indicate that the κ cDNA was over 90% pure by these criteria (2). Furthermore, κ cDNA prepared in the same way from the same κ mRNA preparation was also hybridized to *Eco*RI restriction fragments of mouse DNA separated by size (unpublished data); only a single peak of hybridization, with no background hybridization to other DNA fragments, was obtained. This suggests that the κ cDNA contained no other sequences in appreciable quantities.



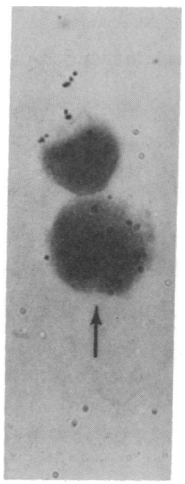


FIG. 4. *In situ* hybridization of κ [$^3$H]-cDNA (24,000 cpm) with ABPL-4 cells. Radioautographs were exposed for 6 weeks. Nucleus of cell indicated by arrow is slightly positive.

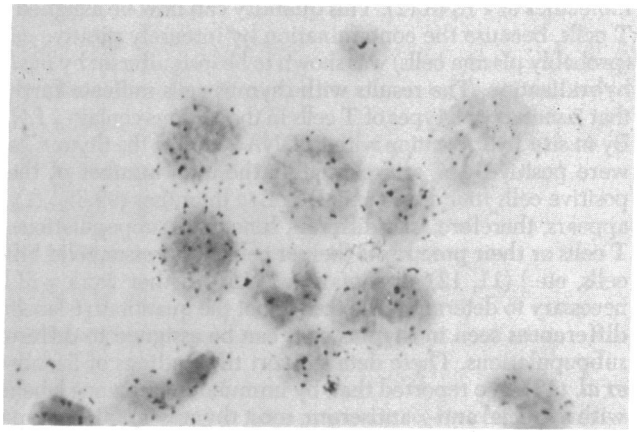


FIG. 5. *In situ* hybridization of κ [$^3$H]cDNA (9600 cpm) with PC 3741 myeloma cells. The radioautograph was exposed for 5 weeks.

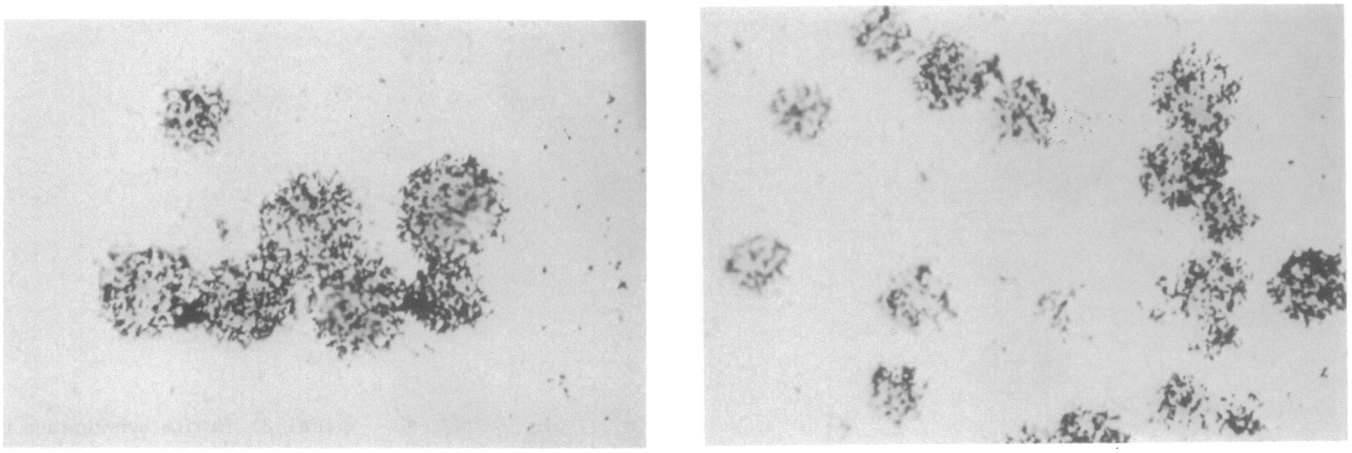


FIG. 6. *In situ* hybridization with 60,000 cpm of PU 5.1 whole cell κ [$^3$H]cDNA. Radioautographs were exposed for 5 weeks. (*Left*) PC 3741 myeloma cells. (*Right*) BALB/c spleen cells, 4 weeks old.

Controls for the specificity of *in situ* hybridization of κ cDNA were provided by the facts that the number of negative cells in the spleen correlated well with the expected number of nonlymphoid cells (Table 1) and that most of the ABPL-4 tissue culture cells that were a population deficient in κ RNA did not hybridize with κ cDNA. That these latter cells were not artifactually unable to hybridize with κ cDNA was demonstrated by a small number of ABPL-4 cells slightly positive in *in situ* hybridization with κ cDNA; the ABPL-4 tissue culture cells had not been cloned and, although the average amount of κ RNA was seven κ RNA molecules per cell (2), they were apparently a mixture of negative and slightly positive cells.

It was further demonstrated that all cells in the spleen and all PC 3741 cells contained hybridizable RNA molecules when tested with a cDNA probe that corresponded to a majority of the mRNA sequences of a cell (Fig. 6). PU 5.1 B lymphoma cells were the source of a whole cell cDNA that reacted to about 90% with the whole cell poly(A)-containing RNAs of EL 4 T cells and of kidney (unpublished data). Because the majority of RNA sequences generally is very similar in different cells (8–10), this whole cell cDNA probe was a useful control. As Fig. 6 shows, not only were all spleen and PC 3741 cells labeled by hybridization, but they also had very uniform grain distributions. These controls demonstrate that the differential labeling found with κ cDNA in thymus and spleen was not an artifact.

Based on these controls, the results of this study clearly indicate that T cells contain significant amounts of κ RNA. By hybridization with RNA from total thymus cells it was found that the average thymus cell contains approximately 1000 molecules of κ RNA (2). This quantity can now be assigned to T cells, because the contamination by intensely positive cells (probably plasma cells) was shown to be insignificant by *in situ* hybridization. The results with thymus cells indicate further that essentially all types of T cells in the thymus contain κ RNA. By *in situ* hybridization with κ cDNA 98.5% of the thymus cells were positive, i.e., approximately the total number of theta positive cells found in young BALB/c thymuses (99.8%) (1). It appears, therefore, that different functional subpopulations of T cells or their precursors (helper cells, suppressor cells, killer cells, etc.) (11, 12) all contain κ RNA. Further work will be necessary to determine whether or not the quantitative labeling differences seen in thymus cells can be assigned to different subpopulations. These data support the findings of Szenberg *et al.* (13) who reported that, by immunofluorescence labeling with a special anti-κ antiserum, most thymus T cells were positive. The present data indicate further that T cells have the potential for κ chain synthesis from endogenous κ RNA.

It appears likely that mature peripheral T cells also contain κ RNA. Mouse spleens contain 80–90% small lymphocytes (6). The percentages of T and B cells are 30–40% and 40–50%, respectively. Because 86% of all spleen cells were positive by *in situ* hybridization with κ cDNA and the average grain counts over the majority of the positive cells in thymus and spleen did not differ, it appears that the average T and B cells contain similar quantities of κ RNA. It remains, however, to be determined whether functionally highly activated T cells increase the synthesis of κ RNA as do plasma cells derived from stimulated B cells.

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