either cell type cultured separately. This enhancement cannot be accounted for by the higher cell concentration of the mixed cell culture, as shown by the results of the following experiment. Either 1.5×10^7 or 2.5×10^7 nucleated spleen cells from sham-operated chimeras were cultured with sheep RBC in 1 ml of medium. The peak response of the 2.5×10^7 cells was never more than 2-fold greater than that of the 1.5×10^7 cells.

The interaction between thymus cells from normal donors and spleen cells from neonatally thymectomized donors is illustrated in Figure 2. As in the radiation chimera experiments, the peak mean number of PFC per dish resulting from the stimulation of normal thymus cells mixed with spleen cells from thymectomized donors was higher and appeared one day later than that produced by the response of normal spleen cells. The response of the mixed cells was at least 70-fold greater than that of either cell type stimulated in separate cell cultures. Results of the control cultures not shown in Figure 2 were very similar to those described in the previous experiment with radiation chimeras.

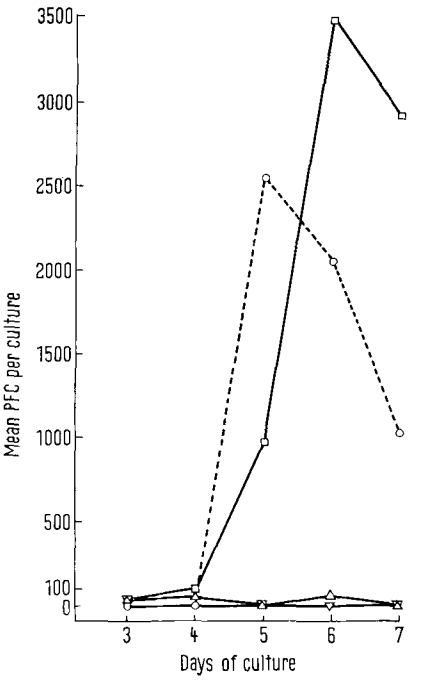

Fig. 2. In vitro primary response to sheep RBC by thymus cells from normal donors and spleen cells from neonatally thymectomized donors. ▽—▽, thymus cells; △—△, spleen cells from thymectomized donors; □—□, thymus cells and spleen cells from thymectomized donors; ○---○, spleen cells from normal donors.

It is worth mentioning that using male DBA/2J or C3HeB/FeJ normal mice thymus cells from 4-week-old donors and bone marrow cells from 12-week-old donors repeatedly failed to produce PFC when stimulated with sheep RBC in mixed or separate cultures, even when as many as 3×10^7 nucleated bone marrow cells (the most that could be obtained from 4 femurs) were cultured with 1×10^7 nucleated thymus cells. The unresponsiveness of this cell mixture suggests that the femural marrow of an adult mouse has too few cells, if any, able to interact with thymus cells in vitro. The interaction observed when spleen cells from thymectomized bone marrow chimeras were used, indicates that bone marrow cells able to interact in vitro or their precursors had proliferated or differentiated to such an extent that enough differentiated cells could be found in the spleen of the irradiated recipients 45 days after bone marrow transplantation. These proliferative and differentiative events can occur in the absence of thymus, as demonstrated by the results with spleen cells from either thymectomized chimeras or neonatally thymectomized mice. In the latter case, the bone marrow origin of the spleen cells able to interact with thymus cells in vitro remains to be determined.

Riassunto. Globuli rossi di pecora inducono in vitro, in colture miste di cellule spleniche di topi timectomizzati e timociti di topi normali, una risposta immune di tipo emolitico paragonabile a quella di cellule spleniche di topi normali. I dati indicano che la risposta immune delle colture miste risulta dall'interazione dell'antigene con timociti e cellule spleniche di origine midollare.

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Molecular Hybridization of RNA from Chicken Spleen Cells after Immunization

Recent studies have shown that new RNA species appear in mouse spleen or peritoneal exudate cells after in vivo[1] or in vitro[2] exposure to erythrocyte antigens. Direct hybridization of labelled RNA and competition experiments indicated that different RNA species appeared with different antigens. The aim of the present work was to confirm these results using chicken spleen cells at various times after immunization with a soluble antigen.

Adult White Leghorn chickens were injected i.v. with 200 mg of human serum albumin (HSA) per 1 kg of body weight. The spleens were excised between the 1st and 5th day after immunization. Spleens were minced by scissors and both washed cells and fragments were suspenedd in Eagle's MEM medium without phosphate. The mixture of fragments and cells from 5–10 chickens was incubated in a total volume of 100–200 ml of medium. P^{32}-labelled phosphate (Na_2HPO_4, carrier free, GmbH Isocomerz, Berlin-Buch) was added to a final concentration of 100 μc per ml. Incubation was stopped after 1 h by cooling in an ice-bath, the cells were collected by centrifugation and frozen.

RNA was isolated from the cells in a mixture of equal volumes of water saturated phenol and 0.05*M* sodium acetate (pH 5.5) containing 0.005*M* EDTA and 0.5% bentonite. Successive extractions were carried out at 25

[1] E. P. Cohen, Proc. natn. Acad. Sci. USA *57*, 673 (1967).
[2] K. Raska jr. and E. P. Cohen, Nature *217*, 720 (1968).

and 65 °C, respectively. The aqueous layers were re-extracted with phenol containing 0.1% 8-hydroxyquinoline and RNA was precipitated by 2.5 vol. of ice-cold ethanol containing 2% potassium acetate. For hybridization RNA fractions prepared both at 25 and 65 °C were used and in general similar results were obtained. The figures given here represent only the data obtained with the RNA extracted at 65 °C, as they were more conclusive and reproducible.

DNA was isolated by MARMUR's technique[3] and the hybridization was performed according to GILLESPIE and SPIEGELMAN[4]. 20 or 30 μg of DNA were fixed to each filter. Radioactivities were determined with the Mark I – Nuclear Chicago liquid scintillation spectrometer. Because the specific activities might differ for the various types of RNA present in our preparations, we have expressed the results of the hybridization in cpm values which have been actually measured.

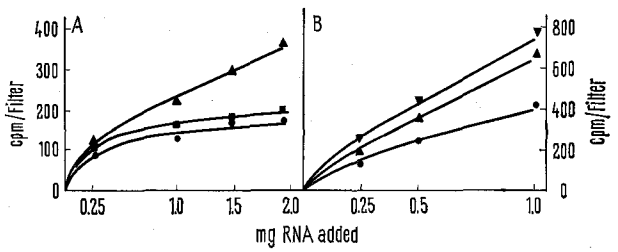
The results of hybridization experiments using RNA from spleens at various times after immunization are given in the Figure. The amount of hybrid forming RNA increased on the 1st and the 2nd day, respectively, after injection of HSA, but on the 5th day values similar to those found for non-immune animals were obtained.

A suspension of spleen cells prepared on the 2nd day of immunization was preincubated for 45 min with 0.1 μg/ml of actinomycin D, followed by 60 min pulse of P^{32}-phosphate. A parallel untreated culture was labelled under the same conditions. RNA isolated from the actinomycin-treated culture hybridized to the same extent or, at high input, even slightly more than RNA labelled without actinomycin pretreatment (Table).

Nuclear RNA extracted from mammalian cells has been reported to be effective in both direct hybridization and competition experiments[5–7]. The nuclear DNA-like RNA may be a polycistronic precursor of mRNA[8] or it may have no direct relationship to cytoplasmic mRNA at all[9]. It has been reported that a considerable part of hybridizable RNA was not transferred to the cytoplasm[6,7]. COOPER[10] has found that the synthesis of rapidly-labelled polydisperse RNA in PHA-stimulated lymphocytes is unaltered by the presence of 0.15 μg/ml of actinomycin D. In our experiments, the hot extraction, rapid labelling (30 min was used in additional experiments) and resistance to actinomycin D would suggest that we were dealing with nuclear RNA. The increase of hybridizable RNA after immunization thus might not represent functional mRNA found on polysomes.

The increase of hybridizable RNA early after immunization (1st and 2nd day) followed by return to normal values on the 5th day has a time course which is dissimilar to the development of the specific antibody response and splenomegaly, with a peak on the 6th day under the conditions of immunization used here[11]. The early onset and cessation of the formation of hybridizable RNA is similar to the stimulation of uridine kinase activity in the spleens of mice after immunization[12]. Synthesis of an actinomycin D-sensitive type of RNA was reported in the early period (2nd day) of antibody response in vitro[13]. The significance of various functional types of RNA in the differentiation of immunocytes remains open for further study. Before one can conclude that gene derepression is occurring at an early stage of immunization, it would be necessary to exclude changes in the specific activities of the hybridizable RNA molecules. However, additional experiments indicated that increased hybridization early after immunization was obtained with RNA labelled for longer time periods when uniform labelling might have already been reached.

During the preparation of this manuscript other evidence for increased hybridization by RNA from the early stage of the immune response in mice was reported[14].



Hybridization of P^{32}-labelled RNA of immune and normal spleen cells. Increasing amounts of RNA extracted at various days after injection of 200 mg HSA per kg body weight were hybridized in duplicate with a mean error of 5%. The specific activities (cpm/μg RNA) of the preparations used are given in parentheses. (A) ● (79), non-immune; ▲ (65), 1st day; ■ (75), 5th day; (B) ● (85), non-immune; ▲ (99), 1st day; ▼ (83), 2nd day.

The effect of actinomycin D on the synthesis of hybridizable RNA

Input of RNA (mg)		0.12	0.24	0.48	1.20	2.40
Cpm per filter	Actinomycin D	66	96	176	359	922
	Untreated	76	100	186	302	702

Spleen cells from chickens on the 2nd day after injection with 200 mg HSA per kg body weight were pre-incubated for 45 min in the presence of 0.1 μg/ml of actinomycin D. Following incubation for another 60 min with P^{32}-phosphate, RNA was isolated. Hybridization was performed in duplicate with a mean error of 10%. Specific activities were 143 and 130 cmp/μg for control and actinomycin D treated RNA preparations, respectively.

Zusammenfassung. Es wird mit neuer Technik gezeigt, dass nach Immunisierung von Hühnern in der Milz neue Ribonukleinsäuren erscheinen.

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[3] J. MARMUR, J. molec. Biol. *3*, 208 (1961).
[4] D. GILESPIE and S. SPIEGELMAN, J. molec. Biol. *12*, 829 (1965).
[5] R. W. SHEARER and B. J. MCCARTHY, Biochemistry *6*, 283 (1967).
[6] R. CHURCH and B. J. MCCARTHY, Proc. natn. Acad. Sci. USA *58*, 1548 (1967).
[7] J. DREWS, G. BRAWERMAN and H. P. MORRIS, Europ. J. Biochem. *3*, 284 (1968).
[8] K. SCHERRER, L. MARCAUD, F. ZAJDELA, I. M. LONDON and F. GROS, Proc. natn. Acad. Sci. USA *56*, 1571 (1966).
[9] G. ATTARDI, H. PARNAS, M. I. H. HWANG and B. ATTARDI, J. molec. Biol. *20*, 145 (1966).
[10] H. L. COOPER, J. biol. Chem. *243*, 34 (1968).
[11] J. CERNY and J. IVANYI, Fol. biol., Praha *12*, 343 (1966).
[12] K. RASKA JR. and E. P. COHEN, Clin. expl. Immun. *2*, 559 (1967).
[13] J. IVANYI, M. MALER, L. WUDL and E. SERCARZ, J. exp. Med. *127*, 1149 (1968).
[14] R. B. CHURCH, U. STORB, B. J. MCCARTHY and R. S. WEISER, J. Immun. *101*, 399 (1968).
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