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Particular RNA Fragments as Promoters of Leukocyte and Platelet Formation in Rabbits

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Abstract. Under well-defined conditions, ribosomal RNA from *Escherichia coli* is fragmented by pancreatic ribonuclease, leading to the appearance of particular RNA fragments. Some of these fragments act as primers for *in vitro* replication of DNA extracted from blood-cell and platelet-forming tissues. In experimental rabbits they restore in a rapid and harmless way normal circulating leukocyte and platelet levels when these have been drastically decreased by various chemotherapeutic agents mainly used in anticancer therapy. Imbalance between polynuclear and lymphocyte count provoked in rabbits by cyclophosphamide can be rapidly corrected by treating the animal with active RNA fragments.

Introduction

It is now well established that replication *in vitro* of an intact single-stranded DNA chain by DNA-dependent DNA polymerase requires the presence of RNA primers in addition to all the necessary components for DNA synthesis (9, 10, 12, 13). This was demonstrated in particular when DNA-dependent DNA polymerase I was used to catalyze this reaction. In previous articles (3, 5, 6), we have shown that specific short-chain RNA fragments obtained from the enzymatic breakdown of ribosomal RNA acted as primers for *in vitro* replication of DNA originating from different sources (5). Since particular RNA fragments are indispensable to DNA *in vitro* replication, we called them *primer RNA.* Their *in vitro* specificity seems to arise from the relative amounts of their component nucleotides (A, G, U, C) which determine their specificity of action: some act on the replication of mammalian DNA, others on that of viral, plant or bacterial DNA. On the basis of these results, we were able to develop methods for stopping the replication of DNA viruses (6) and to induce under axenic condi-

tions the appearance of tumorous cells in *Datura stramonium* grown on synthetic medium *in the presence of plant hormones* (1, 2).

Biologists have made many efforts to obtain specific control over the multiplication of target cells by acting solely on the replication of the genetic material without altering genes and cell structure. In this line of research, there was a possible approach. Many chemotherapeutic agents, particularly antimitotic drugs used for cancer treatment, have a secondary toxic effect on leukocyte and blood platelet stem cells. Could some of our primer RNA act *in vivo* specifically on the DNA of these stem cells, incite them to divide, differentiate and finally restore the normal amount of circulating leukocytes and platelets? This was achieved by the discovery that some RNA fragments were rather selectively incorporated into bone marrow and spleen tissues where platelet and leukocyte formation occurs.

Materials and Methods

Escherichia coli, a harmless host of man's intestinal tract, was used as a source of ribosomal RNA. Nonpathogenic bacteria (T 3000) were grown aerobically at 36 °C (11).

Isolation of Ribosomal RNA. These RNA species were isolated and purified from ribosomes obtained from extracts of *E. coli*, as described elsewhere (4).

Obtaining of RNA Fragments. RNA fragments were obtained by mild degradation of purified ribosomal RNA from *E. coli*, as described elsewhere (7).

Characterization of RNA Fragments. RNA fragments were analyzed and characterized by chemical and physical means. They contain 50 nucleotides and the absorption spectrum of RNA fragments is characteristic of single-stranded ribonucleic acid type molecules. They contain purine nucleotides in excess over pyrimidines (G + A/C + U = 2,3) and are devoid of DNA as trace contaminant.

Reference Substances Used. Poly A, Poly I-Poly C (double-stranded), Poly AG (single-stranded) were obtained from Miles Laboratories.

Antimitotics. Cyclophosphamide (Endoxan) was purchased from Lucien Laboratories and Daunorubicin from Rhône-Poulenc.

Rabbits. Bouska, New Zealand and Fauve de Bourgogne (3–4 kg).

Replication in vitro of DNA. DNA-dependent DNA polymerase I from *E. coli* was partially purified and used as the polymerizing enzyme in the absence and presence of RNA fragments and other components necessary for DNA synthesis (8).

Isolation of DNA from Bone Marrow, Spleen and Brain of Rabbit. DNA was isolated using deproteinizing substances (phenol and chloroform), as described elsewhere (5).

Analysis of Circulating Leukocyte and Platelet Count in Animals Untreated or Treated with Antimitotic Drugs and RNA Fragments. A Coulter Counter was used for leukocyte and platelet count. Polynuclears and lymphocytes were counted separately under the microscope after fixation and staining.

Results

RNA Fragments and Replication of DNA in vitro

In the absence of primer RNA, DNA-dependent DNA polymerase I incubated in complete medium is able to synthesize a very limited amount of acid-precipitable DNA, whose synthesis is determined by measuring the amount of radioactive desoxyribonucleotides incorporated into DNA. When RNA fragments are added to complete medium containing bone marrow or spleen DNA template, DNA replication is very highly stimulated (table I). In the presence of desoxyribonuclease, there is no synthesis of DNA. Remarkable is the observation that RNA fragments stimulate the replication of DNA originating from tissues

Table I. Replication *in vitro* of DNA from various tissues in the absence and presence of RNA fragments

Incubation mixture	^{3}H-TTP incorporated into DNA, cpm								
	10 min			20 min			30 min		
	DNA 1	DNA 2	DNA 3	DNA 1	DNA 2	DNA 3	DNA 1	DNA 2	DNA 3
Complete	550	451	466	662	520	507	701	580	660
+ RNA frag. (4 µg)	1,980	1,276	506	2,164	1,476	617	2,256	1,634	756
+ RNA frag. (4 µg) + DNase (2 µg)	112	104	110	123	126	98	130	–	–
+ RNA frag. (4 µg) – DNA	102	107	98	133	112	108	140	–	132

Incubation medium contains per 0.15 ml: Tris-HCl buffer, pH 7.65: 25 µmol; $MgCl_2$: 2 µmol; 4 d-XTP: each 5 nmol (+ ^{3}H-TTP: 50,000 cpm); DNA: 0.2 µg; RNA fragments: 4 µg; DNA-dependent DNA polymerase: 80 µg (8). Incubation 10, 20 and 30 min at 36 °C. Trichloroacetic-acid Whatman-precipitable material is filtered on a GF/C glass filter, washed, dried and radioactivity measured in a Packard liquid spectrometer. DNA 1 = Bone marrow; DNA 2 = spleen; DNA 3 = brain.

where leukocytes and platelets are formed, while these same fragments are without an effect on replication of DNA from brain and kidney. These results suggest that *in vivo* RNA fragments are able to promote replication of DNA of the stem cells and consequently accelerate their division process.

In vivo *Action of RNA Fragments on White Blood Cell and Platelet Formation*

Activity of RNA fragments in leukopoiesis and platelet formation was tested in white (Bouska) 3- to 4-kg rabbits receiving various immunodepressive drugs. When the leukocyte count had considerably fallen, RNA fragments had a dramatic restoring action on leukopoiesis. For instance, in a rabbit which had received a daily injection of 75 mg cyclophosphamide (= 100 mg Endoxan) for 8 consecutive days, the leukocyte count had fallen from 11,000 to 4,000; with a single intravenous injection of 5 mg of RNA fragments, the leukocyte count was back to normal within 24 h. Uninterrupted immunosuppressive treatment of rabbits for 2–3 months with very high doses (75 mg/rabbit/day) of cyclophosphamide is normally lethal within 8–12 days; but a single weekly injection of 5 mg specific RNA fragments proved sufficient to restore normal leukopoietic activity and keep the animal in good health (fig. 1). Rapid stimulation of platelet formation (table II) followed by stabilization of platelet count within normal physiological limits was also obtained when RNA fragments were given to rabbits receiving drugs such as Daunorubicin (which decreases the number of blood platelets as well as that of leukocytes) and to control rabbits (not shown).

Table II. Effect of BLR on platelet formation in rabbits treated with Daunorubicin

	Days												
	1	2	3	4	5	6	8	9	10	11	12	13	14
Daunorubicin	+	+	+	+ (+ BLR)	–	–	+ (+ BLR)	–	–	–	–	–	–
Platelet count $\times 10^{-3}$	395	330	–	290	63	29	15	35	36	40	80	150	460

Daunorubicin: 5 mg, i.v.; BLR: 5 mg, i.v. and 20 mg *per os*. Rabbits treated with only Daunorubicin die within 6–9 days.

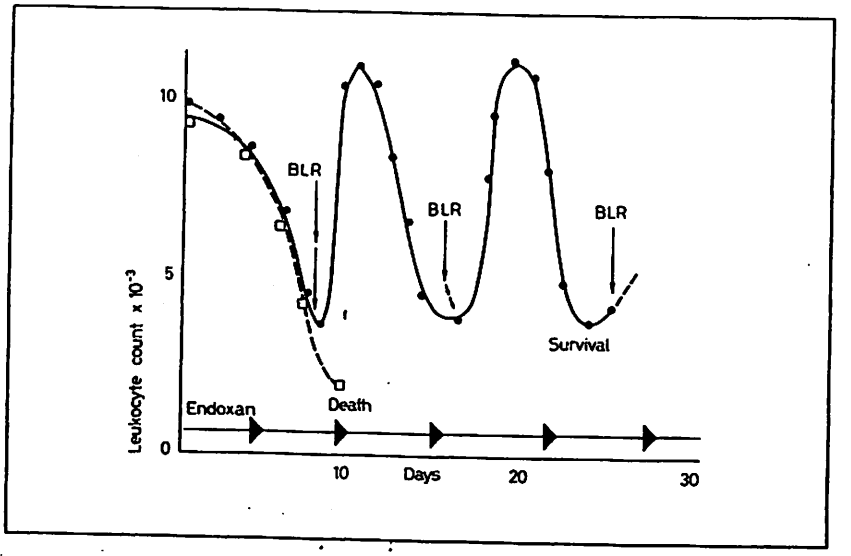

Fig. 1. RNA fragments (Beljanski Leukocyte Restorers; BLR) acting as restorers of leukocytes in a rabbit permanently treated with cyclophosphamide. The control rabbit which received 75 mg of cyclophosphamide i.v. every day died. The rabbit also treated with cyclophosphamide, but receiving BLR (5 mg) i.v. (arrows) survived. The leukocyte count was determined using a Coulter Counter.

Specific RNA Fragments Restore the Balance between Lymphocytes and Polynuclears

It is well established that cyclophosphamide injected to animals or humans decreases not only the total number of circulating leukocytes but even more drastically that of polynuclears, which decreases from 55 to 18%. Thus an imbalance between polynuclears and lymphocytes appears in circulating white blood cells of rabbits permanently treated with cyclophosphamide. In man, cyclophosphamide (Endoxan) and several other drugs bring about a similar imbalance, with lymphocytes remaining largely predominant (around 80%).

When cyclophosphamide-treated rabbits are given RNA fragments intravenously polynuclear and lymphocyte counts increase in a differential way so that the normal balance is restored within 4–5 h after injection of RNA fragments. 6 or 7 days later, the action of RNA fragments ceases and the cyclophosphamide-induced imbalance reappears; it can be corrected again with a new RNA fragment injection. In 1 cyclophosphamide-treated rabbit, we compared RNA fragment activity with that of poly AG and poly A (fig. 2); neither

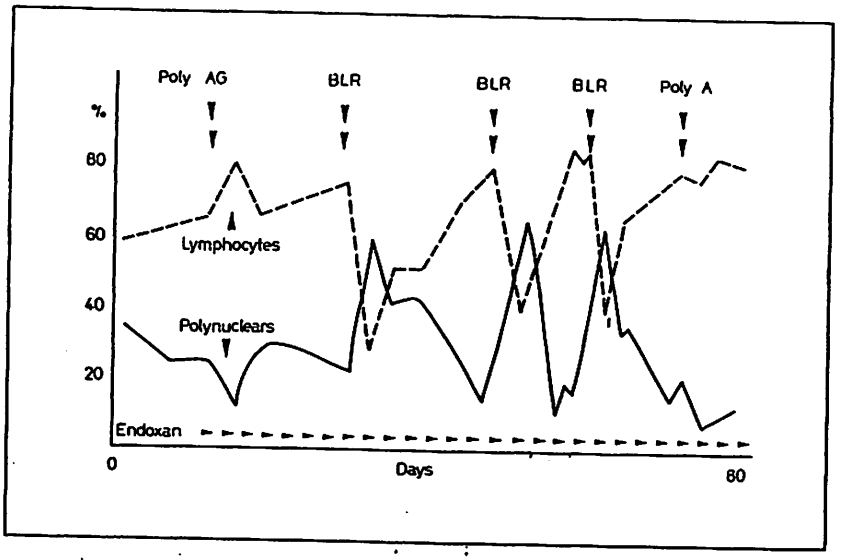

Fig. 2. RNA fragments restore the balance between lymphocytes and polynuclears. A 3-kg rabbit was treated daily with 75 mg of cyclophosphamid i.v. When the leukocyte count had fallen and polynuclear and lymphocyte imbalance appeared, Poly AG (3 mg), BLR (5 mg), and Poly A (5 mg) were given i.v. at the intervals shown.

was able to stimulate leukocyte formation or restore lymphocyte/polynuclear balance. On the basis of these results, one is inclined to consider that RNA fragments are naturally occurring substances which are used by living organisms to restore normal leukocyte and platelet levels after drug damage. It should be emphasized that RNA fragments once degraded with alkali completely lose their *in vitro* and *in vivo* activity.

Discussion and Conclusions

The data presented here show that RNA fragments which consist of short single-stranded polyribonucleotide chains devoid of DNA and protein contaminants, obtained by enzymatic breakdown of *E. coli* ribosomal RNA, exert an unquestionable and rapid stimulating action on white blood cell and platelet formation in the rabbit. These results were expected because the same RNA fragments act *in vitro* as primers for replication of DNA extracted from mammalian spleen and bone marrow, i.e. tissues which contain leukocyte and platelet

stem cells. In addition, this expectation was also strengthened by the observation that labeled RNA fragments when injected into a rabbit are seen to concentrate in the bone marrow and spleen.

Permanent treatment of rabbits with very high doses of cyclophosphamide (75 mg/day/rabbit) 'destroys' the stem cells, thus suppressing leukocyte formation, and causes the animals to die when the leukocyte count becomes too low. The fact that RNA fragments are able to normalize the leukocyte level of cyclophosphamide-treated animals suggests that these fragments have a high affinity for normal stem cells in which they promote the replication of DNA, i.e. the first step of cell division. However, one could object that RNA fragments probably act by liberating in some way leukocytes stocked in blood vessels and some tissues. If this were so, one would expect that in a rabbit permanently treated with cyclophosphamide, several consecutive administrations of RNA fragments would exhaust the leukocyte stock, thus creating a situation in which the animal would die. This is not the case, as demonstrated by the following experiment: rabbits receiving daily 75 mg of cyclophosphamide for 6–8 weeks and repeated doses (16–20) of the RNA fragments survive and always respond to new injections of RNA fragments. Data obtained in the above experiment show that there is no cumulative effect of the specific RNA fragments and that the fragments injected do not lead to loss of ability of rabbits to respond to the leukopoietic activity of the RNA fragments. In itself this experiment strongly suggests that specific RNA fragments act on stem cells, although this should be demonstrated, using *in vitro* stem cells and RNA fragments. One particular feature of RNA fragment activity is their rapid correction of the imbalance between polynuclears and lymphocytes occurring in rabbits permanently treated with cyclophosphamide. This observation suggests that the RNA fragments might interfere not only by acting on the division process of stem cells or their derivatives giving rise to polynuclears, but also that they could act (directly or indirectly) on the maturation of polynuclears. If so, one might explain the rapid restoration of the polynuclear/lymphocyte balance on one hand and the increase of the respective levels of polynuclears and lymphocytes on the other. However, it still remains to be shown in a direct way on which type of cells the RNA fragments exert their activity. This problem is under investigation.

Although it is unquestionable that RNA fragments strongly increase platelet count in rabbits treated with Daunorubicin, we have to demonstrate in a precise way whether they act on megakaryoblasts which are transformed by endomitosis into giant cells (megakaryocytes) from which platelets originate through a fragmentation process. But it is an established fact that the RNA fragments used

in this work exhibit a remarkable biological activity in the formation of leukocytes and platelets in rabbits treated with antimitotic drugs.

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