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Poly(A)·Poly(U) as Adjuvant in Cancer Treatment Distribution and Pharmacokinetics in Rabbits (42082)

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Abstract. Rabbits were injected with poly(A)·poly(U) and the metabolic fate and stability studied using scintigraphic techniques, radioactive counting, and subcellular fractionation. The complex has a very long lifetime (measured in days) in both liver and spleen, the major locations of concentration in the animal, with about nine times more per gram of tissue in the spleen compared with the liver. In spleen cells the material is about equally divided between nuclei and cytoplasm. The polymer is slowly degraded to shorter molecules in these cells but, even 1 week after injection, significant amounts of apparently intact polynucleotide complex can be detected in both cellular fractions.

Polyadenylic·polyuridylic acid poly(A)·poly(U), a double-stranded complex of synthetic polyribonucleotides, has shown significant antitumor effects in a number of tumor systems: as an adjuvant to surgery (1) in spontaneous mammary tumor and transplanted hamster melanoma, in Rausher MuLV-induced BALB/c mouse ascites (2), and as prophylactic therapy in spontaneous AKR mouse leukemia (3) and in spontaneous C_3H/He mouse mammary tumor (4). A synergistic inhibition of established transplantable mammary tumor in C_3H/He mice by combined treatment with poly(A)·poly(U) and cyclophosphamide has also been reported (5). Recently, in a randomized therapeutic trial on 300 operable breast cancer patients, a significant beneficial effect was observed in patients treated with the complex (6, 7). No toxic symptoms could be evidenced (8) in mice injected with very high doses (250 mg/kg) of poly(A)·poly(U).

The mechanisms governing the antitumor action of poly(A)·poly(U) have been discussed with respect to various biological effects such as immunomodulation for both humoral and cell-mediated immune response (9, 10), induction of interferon in rabbits (11), mice, and humans (12), and enhancement of natural killer (NK) cell activity (5). Few *in vitro* studies have been reported on the cellular properties of the complex. Shell (13) has demonstrated that poly(A)·poly(U) penetrated Ehrlich ascites tumor cells, without separation of the two strands. Fenster *et al.* (14) have shown that uptake of the complex by mouse ascites tumor cells and by human lymphocytes in culture occurred and that the material migrated rapidly into the cell nucleus and remained intact for 2 hr. In contrast, Mutchnich *et al.* (15) presented evidence indicating that 80% of thymocytes bound poly(A)·poly(U) on the cell membrane *in vitro*. However, no studies on the organ localization, cellular penetration, and metabolic lifetimes of poly(A)·poly(U) (or other polynucleotide complexes) after injection into animals of any kind (including man) have been reported.

We now present *in vivo* results on the fate of poly(A)·poly(U) after injection into rabbits as a complement to earlier studies with cell cultures which do not always reflect observations obtained with living animals.

Materials and Methods. *Polynucleotides.* Poly(A)·poly(U) was prepared as previously described (16). The material had a mean sedimentation constant of $s_{w,20} = 10.01$ with an average molecular weight of about half a million, but was polydisperse with polynucleotide complexes ranging from 250,000 to several million daltons. These preparations are stable for more than $2\frac{1}{2}$ years when stored as a sterile solution in physiological saline at 4°C.

^{51}Cr-labeled poly(A)·poly(U). A solution containing 2 mCi of $^{51}Cr^{3+}$ (NEN, sp act 72.23 mCi/mmole) in 1 ml of 0.1 *N* HCl was adjusted to pH 7 with 1 ml of 0.1 *N* NaOH and 0.1 ml of 0.1 *M* Tris–HCl, pH 8.2, and

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was added to a solution containing 800 μg of poly(A)·poly(U) in 2 ml of 0.1 *M* NaCl, 0.01 *M* Tris, pH 7.4, 0.3% sodium dodecyl sulfate (SDS). After 30 min of incubation at 66°C, NaCl was adjusted to 0.3 *M* and the solution was incubated at 37°C for 8 hr and then left at 4°C overnight.

Separation of polynucleotides from free $^{51}Cr^{3+}$ was obtained by passage of the solution through a Sephadex 100 column (30 × 1 cm) in 5×10^{-3} *M* Tris, pH 7, 0.1% SDS using the same buffer for elution. Appropriate fractions were pooled and the OD at 260 nm and radioactivity (RA) were measured. The specific RA was between 1×10^5 and 2×10^5 cpm/μg for different preparations.

^{51}Cr-labeled poly(A)·poly(U), 16–18 S. Labeled poly(A)·poly(U) was precipitated in 0.2 *M* NaCl with 2 vol of ethanol, redissolved in 0.15 *M* NaCl, 0.01 *M* Tris, pH 7.4, 0.1% SDS, and centrifuged on a 5–20% sucrose gradient at 20,000 rpm for 20 hr at 17°C. The ^{51}Cr-labeled poly(A)·poly(U) fractions of 16–18 S were pooled and precipitated with 2 vol of ethanol.

Subcellular studies. About 3×10^7 cpm of ^{51}Cr-labeled poly(A)·poly(U) was mixed with 1500 μg of unlabeled poly(A)·poly(U) in physiological saline and the mixture was injected intravenously in adult rabbits at a dose of 500 μg of poly(A)·poly(U)/kg.

Rabbits were sacrificed after 90 min, 24 hr, 76 hr, and 7 days and the spleens and liver collected. Male New Zealand white rabbits weighing 3 to 3.5 kg were used (age 100–120 days) for these studies and for scintigraphy.

Nuclear and cytoplasmic extracts. The organs were frozen in liquid nitrogen, then homogenized for 20 sec three times in a Waring blender. Tissue homogenates were lysed in the presence of 0.5% Nonidet P-40 in 0.01 *M* Tris HCl, pH 7.4, 0.2 *M* KCl, 0.02 *M* $MgCl_2$, 0.25 *M* sucrose at 0°C, and the degree of liberation of nuclei was controlled with a phase contrast microscope every minute. The lysis was usually complete within 10 min. Nuclei were separated by centrifuging 15 min at 3000*g* at 0°C and the supernatant was collected.

Nuclei were suspended in 0.01 *M* Tris HCl, pH 7.4, NT buffer, 0.015 *M* NaCl, 0.1% SDS, and 200 μg/ml of Pronase (Sankyo). The mixture was left 20 min at room temperature; then 1% SDS was added and the solution was extracted with phenol–chloroform–isoamyl alcohol (1/1/24, v/v) 10 min at 4°C and then centrifuged for 10 min at 10,000*g*. A mitochondria-free cytoplasmic phase was obtained by centrifuging the supernatants for 20 min at 12,000*g*. They were then adjusted to 0.2 *M* NaCl, 0.1% sarcosyl, and 200 μg/ml of Pronase, left for 30 min at room temperature, and extracted with phenol–chloroform–isoamyl alcohol followed by centrifugation at 10,000*g* for 20 min, and the supernatant was collected.

Scintigraphy. Preparations of poly(A)·poly(U) (material used for clinical application) containing 120 to 160 μCi of ^{51}Cr label (in physiological saline) were injected intravenously into the ear vein of rabbits, using injection of Nembutal for anesthesia. Distribution of the ^{51}Cr-labeled poly(A)·poly(U) after injection into the animal was followed with an Angar (Nuclear Enterprises) scintillation camera (useful diameter, 26 cm) equipped with a specially made pinhole-type collimator. This type of collimator allows enlargement or reduction of the resultant image depending on the distance from the subject. The effective field and sensitivity are inverse functions of the enlargement which is taken into account for counting. The scintillation camera is coupled to an informatic system (Scinti 16, Informatek) composed of a computer (31 K bits, T 1600 Télémécanique), two memory storage magnetic disks (2 × 5 M each, Control Data 9427) for treatment of the data, and a graphic display terminal (Tektronix 4012 screen and 4631 dry photocopier) for visual presentation of the output. The numeric treatment of scintigraphic data provides results in the form of analogic images, as curves of total activity in a given organ with time, isocount contours, activity in a given defined zone, or as ratios of activity between two defined volumes. These data can be obtained at given time intervals or in a continuous fashion.

Results. Scintigraph studies were performed in rabbits injected with ^{51}Cr-labeled poly(A)·poly(U) or with the same amount of free $^{51}Cr^{3+}$. Representative printout images at different times after intravenous injection are shown in Figs. 1–3. It can be seen (Fig.

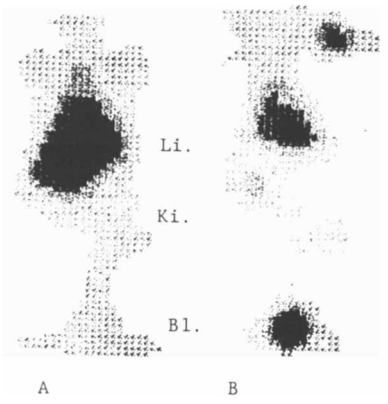


FIG. 1. (A) Localization of ^{51}Cr-labeled poly(A)·poly(U) 15 min after injection. (B) As for A but after injection of the same amount of free $^{51}Cr^{3+}$. Li, liver; Ki, kidney; Bl, bladder.

1A) that after 15 min the highest concentration occurs in the liver in rabbits injected with ^{51}Cr-labeled poly(A)·poly(U). In contrast, maximal concentration appears in the urinary bladder area in rabbits injected with free ^{51}Cr, although the liver and the kidneys are clearly visible (Fig. 1B). One hour after injection of labeled poly(A)·poly(U) the highest concentration is still in the liver. However, a part of the activity is released and concentrated in the bladder (Fig. 2A). With free ^{51}Cr, concentration in the liver is decreased and maximum concentration is in the bladder (Fig. 2B). Twenty-four hours after injection the major concentration of poly(A)·poly(U) is still in the liver; the urinary bladder concentrations are no longer visible (Fig. 3A). In contrast, with free ^{51}Cr the highest concentration is still in the bladder (Fig. 3B). Calculated decay times to 50 and 25% of maximum radioactivity in the liver were 4 and 9 days, respectively, for poly(A)·poly(U). The results are representative of four rabbits which showed identical behavior within the limitations of the technique.

A second series of rabbits was injected intravenously with about 3×10^7 cpm of 16–18 S ^{51}Cr-labeled poly(A)·poly(U) mixed with approximately 1500 μg of unlabeled poly(A)·poly(U) and the radioactivity of aliquots from liver and spleen was determined at different times. As shown in Table I the major part of the labeled poly(A)·poly(U) was incorporated into the liver within 90 min. Although only a minor portion of the complex was located in the spleen, the amounts incorporated were proportionally about nine times higher in the spleen than in the liver, when expressed per gram of tissue.

Nuclei and cytoplasm of spleen cells were prepared, and as shown in Table II the polynucleotide complex was almost equally distributed between the cytoplasmic and nuclear fractions. The sucrose gradient sedimentation profiles (Figs. 4–7) indicate that in spleen cells the complex of size 16–18 S remained undegraded during the first 90 min following inoculation compared to the control. Shorter fragments appear after 24 hr and increased in the course of time. However, even after 7 days a significant proportion

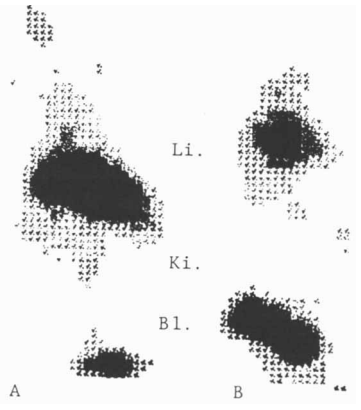


FIG. 2 (A, B). As for Fig. 1 but 1 hr after injection.

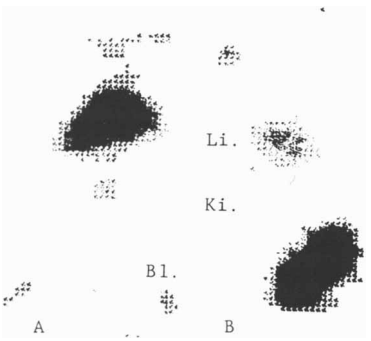


FIG. 3 (A, B). As for Fig. 1 but 24 hr after injection.

TABLE I

	Weight of total radioactivity			
	Organ (g)	Incorporated (cpm)	% of the input radioactivity	Radioactivity per g of tissue
Liver				
1	180	6,800,000	61.8	37.800
2	191	6,200,000	59.6	32.400
Spleen				
1	1.12	388.000	3.5	346.500
2	1.14	398.400	3.83	349.500

Note. cpm injected (1) 1.1×10^7; (2) 1.04×10^7.

(Table II) still sedimented with the same coefficient as the intact poly(A)·poly(U). Uptake in the spleen may be confined to macrophages or other adherent cells, but given the lack of specificity of attachment of poly(A)·poly(U) for cells in culture (see introduction) this seems unlikely.

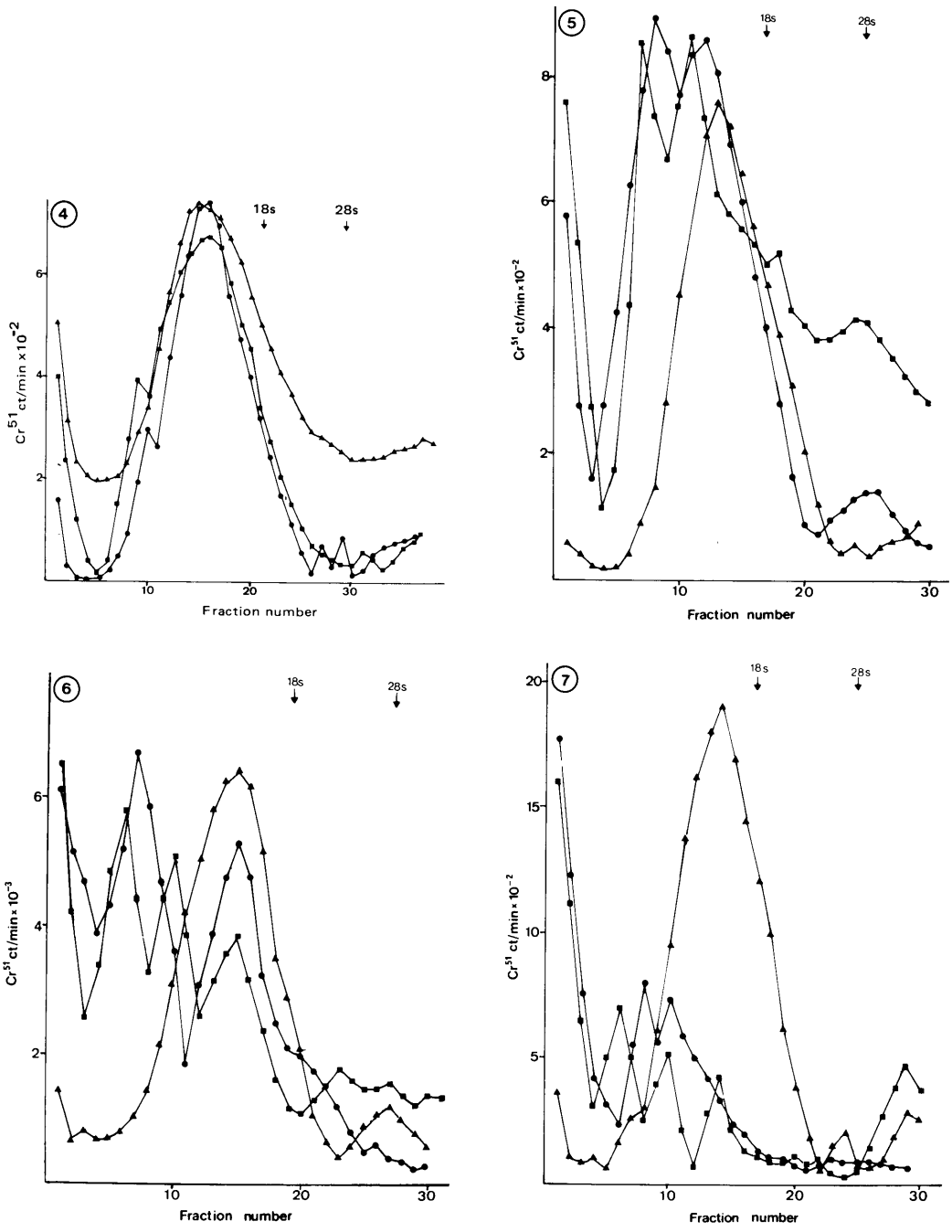
The ^{51}Cr-labeled poly(A)·poly(U) does not lose or exchange the label under physiological conditions of pH and temperature. That the label is not liberated in the *in vivo* experiments followed by reincorporation into other polymers is shown by (a) comparison with free ^{51}Cr; (b) retention of the label with a 16–18 S fraction in sedimentation for up to a week, with slow production of labeled molecules of lower molecular weight; (c) if degradation to Cr-labeled monomers occurs these would not be substrates (and rather act as inhibitors) for various polymerase systems and hence could not be reincorporated into nucleic acids.

Discussion. The capacity of poly(A)·poly(U) to inhibit the growth of some types of neoplasia in rodents is well established (1–5). The beneficial effect on tumor inhibition in breast cancer in humans (6, 7) and the absence of any toxic effects confer a special interest to pharmacological studies of this complex.

Scintigraphic examination of organs of rabbits injected with ^{51}Cr-labeled poly(A)·poly(U) showed an unexpected metabolic stability of the double-helical polynucleotide. Maximal amounts are in the liver and the decay times to 50 and 25% of maximum radioactivity were 4 and 9 days, respectively. In contrast the elimination of free ^{51}Cr was relatively rapid and 24 hr after the injection the majority of activity was concentrated in the bladder (Fig. 3B). Direct determination of the radioactivity in the liver and in the spleen 90 min after injection of ^{51}Cr-labeled poly(A)·poly(U) showed that 61.8% of the input radioactivity was incorporated into the liver. Although only 8.5% of the total radioactivity was located in the spleen, the amount incorporated per gram of tissue was about

TABLE II. DISTRIBUTION OF POLY(A)·POLY(U) IN CYTOPLASMIC AND NUCLEAR FRACTIONS OF SPLEEN CELLS AS A FUNCTION OF TIME

Time after injection	% of input radioactivity in spleen	% Radioactivity in nuclei	% Radioactivity in 12–18 S fraction of nuclei	% Radioactivity in cytoplasm	% Radioactivity in 12–18 S fraction of cytoplasm
90 min	3.82 ± 0.1	47.0 ± 1.4	90.0 ± 0.9	53.0 ± 1.4	93.0 ± 1.4
24 hr	3.15 ± 0.03	48.4 ± 0.6	45.7 ± 1.2	51.6 ± 0.7	47.9 ± 1.5
76 hr	2.30 ± 0.1	45.9 ± 0.9	29.6 ± 1.2	54.1 ± 0.9	36.3 ± 1.9
7 days	0.62 ± 0.05	44.6 ± 0.8	21.1 ± 1.5	55.4 ± 0.9	33.2 ± 2



FIGS. 4–7. Sucrose gradient centrifugation of ^{51}Cr-labeled poly(A)·poly(U) of nuclear and cytoplasmic spleen extracts. Rabbits received an intravenous injection of about 3,500,000 cpm/kg and were sacrificed 90 min (Fig. 4), 24 hr (Fig. 5), 72 hr (Fig. 6), and 7 days (Fig. 7) later. Aliquots of the cytoplasmic and nuclear spleen extracts were layered onto 5–20% sucrose and centrifuged at 20,000 rpm for 20 hr at 17°C in a SW 27 rotor of a Beckman ultracentrifuge. Fractions were collected and processed for acid precipitation and counting. The vertical arrows indicate the 18 and 28 S rRNA peaks in the cytoplasmic extracts as determined by measuring the absorption at 260 nm of the corresponding fractions. Control poly(A)·poly(U), ▲; cytoplasmic extracts, ●; nuclear extracts, ■.

nine times higher than in the liver. Sucrose gradient sedimentation profiles indicate that during this time the complex remained undegraded in spleen cells compared to the profile of intact 16–18 S poly(A)·poly(U). This result is not altogether unexpected since an *in vitro* study in tumor lymphocytes had earlier shown that poly(A)·[^{3}H]poly(U) remains intact for at least $2\frac{1}{2}$ hr (14). The present study conducted *in vivo* shows that shorter fragments of poly(A)·poly(U) appear 24 hr after inoculation and the amount increases over 7 days. Nevertheless, even after 1 week a nonnegligible quantity remains undegraded in the cytoplasm and in the nuclei of spleen cells.

This somewhat surprising *in vivo* metabolic stability of poly(A)·poly(U) may well have a bearing on the biological activities of this polynucleotide complex. Thus poly(A)·poly(U) acts on T cells and after a single injection of 300 μg in A/Sn mice the proportion of T cells in the spleen rose to a peak the 3rd day after injection and represented about 80% of the total cell population. By the 4th day after injection of poly(A)·poly(U) the percentage of T cells began to decrease but normal values were not reached until the 15th day (17). A boosting effect of the complex on natural killer (NK) cell activity has been observed in mice (5) and men (16). In mice kinetic studies showed that the effects occurred rapidly (within 24 hr) and persisted for several days after injection. Treatment with poly(A)·poly(U) also leads to an enhancement of 2–5 A synthetase, an enzyme marker for the production and action of interferon in both man and rodents (16, 18). Poly(A)·poly(U) seems to act to various extents on all of the cells involved in humoral and cell-mediated immune responses (10), including natural killer cell activity (5), and in the induction of interferon (11, 12). All these biological activities are thought to play a role in the antitumor mechanism of this nontoxic complex. Moreover, an indication of the direct suppressive effects of poly(A)·poly(U) on neoplastic cells evidenced as an alteration in the cyclic adenosine 3′5-monophosphate system has been reported (2), and it has been recently observed that tumor cells themselves produce interferon in response to intravenous injection of poly(A)·poly(U) in mice (19). However, the relevance of the long-lasting persistence of undegraded poly(A)·poly(U) in the liver and the spleen to antitumor effects observed both in experimental animal models and in humans remains to be determined. From a practical viewpoint the findings justify (a posteriori) the clinical schedule (injection once a week) used for the treatment of human patients.

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