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The Effect of Synthetic Double-Stranded Polyribonucleotides on Haemopoietic Colony-Forming Cells *in vivo*

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Summary. Intraperitoneal inoculation of 100 μg Poly I–Poly C resulted in an an eight-fold increase in spleen colony forming cells by the sixth day after inoculation. The same dose of Poly AU resulted in a negligible cellular response and no increase in the level of colony stimulating factor in the serum. The colony stimulating activity of serum after inoculation of Poly I–Poly C was increased, but assay of this increase in early sera was complicated by the presence of a colony inhibitor. Some properties of inhibitor-containing sera were investigated and consideration given to the possibility that the colony inhibitor is related to interferon.

INTRODUCTION

Previous experiments have shown that injection of a variety of antigens and adjuvants into mice caused an increase in the number of colony forming cells (granulocyte-macrophage progenitor cells) in haemopoietic tissues (McNeill, 1970c). It was also shown that some of these antigens in association with a serum α-macroglobulin can cause an increase in the responsiveness of colony forming cells (CFC) to colony stimulating factor when added to cultures of normal bone marrow cells *in vitro* (McNeill, 1970a, b). There was, however, no obvious correlation between the capacity of an antigen to cause colony potentiation *in vitro* and its ability to stimulate a CFC response *in vivo*, and it was suggested that the effect *in vivo* was mainly due to stimulation of the CFC system by colony stimulating factor released in response to the injected materials. These observations have been extended to study the effects of synthetic double-stranded polyribonucleotides Poly I–Poly C and Poly AU on colony formation *in vitro* (McNeill, 1971) and it has been shown that these complexes can also potentiate colony formation, the effect being dependent upon the double-stranded nature of the molecules. The action of polynucleotides was less complex than that of antigens in this respect since it did not require the α-globulin co-factor to cause colony potentiation. In the light of this finding and the observation that Poly AU was more effective than Poly I–Poly C in terms of the concentration required to cause colony potentiation, a comparison of their activity *in vivo* should give more information on the possible contribution of the potentiation effect to CFC responses *in vivo*. An additional reason for investigating the activity of Poly I–Poly C *in vivo* was that it is known to be a potent inducer of interferon. Biochemical investigation of colony stimulating factor (Stanley, Robinson and Ada, 1968; Stanley and Metcalf, 1969) has revealed certain

similarities to interferon such as stability at low pH, and it is conceivable therefore that these substances belong to the same general class of biologically active proteins.

MATERIALS AND METHODS

Polynucleotides. Poly I–Poly C and Poly AU were obtained as desalted lyophilized products from P. L. Biochemicals Inc., Wisconsin. Both were reconstituted in sterile normal saline at a concentration of 1 mg/ml and stored at 4°.

Freund's complete adjuvant was obtained from Difco. *Sh. flexneri endotoxin* was supplied by Dr D. A. R. Simmons. *Influenza virus* (MEL strain) was grown in the allantoic cavity of fertile hens eggs. The amount of virus was measured by a conventional haemagglutination titration using a perspex cavity plate. The number of haemagglutinating units (HAU) was expressed as the highest dilution of allantoic fluid which resulted in complete agglutination of the erythrocyte suspension.

Mice. All experiments were performed on 2–3-month-old female C_{57}BL mice from an inbred colony maintained in this Department. To determine the CFC responses to polynucleotides groups of mice were inoculated intraperitoneally at intervals over a 14-day period. When a series was complete all mice were killed by cervical dislocation and spleen and bone marrow removed for culture.

Cultures. The methods of preparing cell suspension, the media and methods of culture have been described previously (McNeill, 1970c, 1971).

Serum colony stimulating activity. Serum pools were made from groups of five mice bled at intervals after intraperitoneal inoculation of polynucleotide. Mice were bled from the axillary vessels under ether anaesthesia, blood from mice within each group pooled, the serum separated and stored at −20°. Colony stimulating activity of each serum pool was estimated by incorporating several doses of serum in base layers of Eagle's medium containing 1·2 per cent agar on to which were placed 1 ml aliquots of a suspension of normal bone marrow cells (5×10^4 per ml) in Eagle's medium containing 0·3 per cent agar. All sera from any series were assayed in the same set of cultures and the number of colonies stimulated by each dose expressed as a percentage of the number stimulated by the same dose of a standard normal C57BL serum which was used throughout the experiments. Sera were usually assayed in duplicate cultures for each dose.

Serum interferon assay. Serum dilutions were made in Eagle's medium and L-cell monolayers in 50 mm plastic dishes incubated at 37° overnight with 2 ml of these dilutions. Following this the monolayers were thoroughly washed with Eagle's medium and infected with 30 plaque forming units of encephalomyocarditis virus in 0·2 ml medium. Virus was absorbed for 2 hours at 37°, the inoculum removed and the cells overlaid with Eagle's medium containing 1·25 per cent Agar-Noble (Difco) and 5 per cent foetal calf serum. Plaques were counted after incubation at 37° for 2 days. Each dilution of serum was tested on duplicate plates and three or four two-fold dilutions used in each titration. The interferon titre was expressed as that dilution which inhibited 50 per cent of plaques.

RESULTS

1. COLONY FORMING CELL RESPONSES

Spleen. Preliminary results showed that intraperitoneal inoculation of 5 μg Poly I–Poly C caused a negligible increase in spleen CFC. Fig. 1 shows the increase in spleen

CFC following intraperitoneal inoculation of 50 μg of either Poly I–Poly C or Poly AU. Each point is the average of results from three to six mice and is expressed as the increase in CFC per 10^6 spleen leucocytes over the number in control spleen suspensions cultured at the same time. Since variation in the sensitivity of culture conditions from experiment to experiment can affect the absolute number of colonies obtained, amalgamation of results from separate experiments depends on a common baseline. These results show that Poly I–Poly C was more effective than Poly AU which gave a negligible response. One

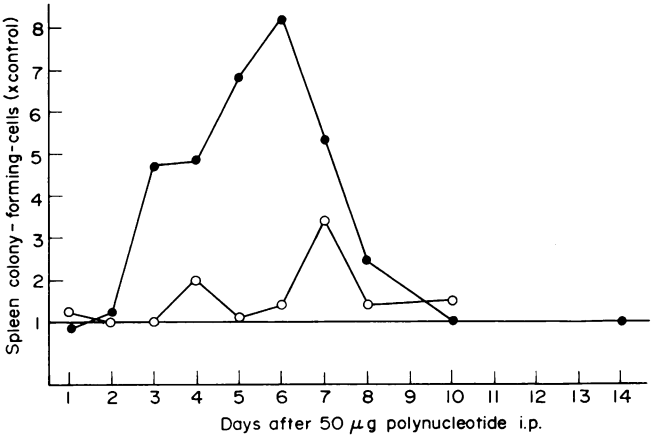

FIG. 1. Colony forming cell responses in spleen following i.p. inoculation of 50 μg (●) Poly I–Poly C, or (○) Poly AU. Increase expressed as a function of the number of colony forming cells in spleens of control mice.

interesting feature of the CFC response to Poly I–Poly C was its delay until the third post-inoculation day.

Bone marrow. Table 1 shows the number of CFC per femoral shaft marrow during the 5 days following Poly I–Poly C and on the fifth day after Poly AU. This shows that the inoculation of polynucleotides did not cause any significant change in marrow CFC.

TABLE 1

COLONY FORMING CELLS PER FEMORAL MARROW FOLLOWING I.P. INOCULATION OF 50 μg POLY I–POLY C OR POLY AU

		Colonies per femoral marrow (x control)
Poly I–Poly C	Day 1	0·8
	Day 2	1·3
	Day 3	1·4
	Day 4	1·1
	Day 5	1·2
Poly AU	Day 5	1·1

2. SERUM COLONY STIMULATING FACTOR

Fig. 2 shows the activity of serum pools obtained from groups of mice bled at intervals after intraperitoneal inoculation of 100 μg Poly I–Poly C or Poly AU. The activity is

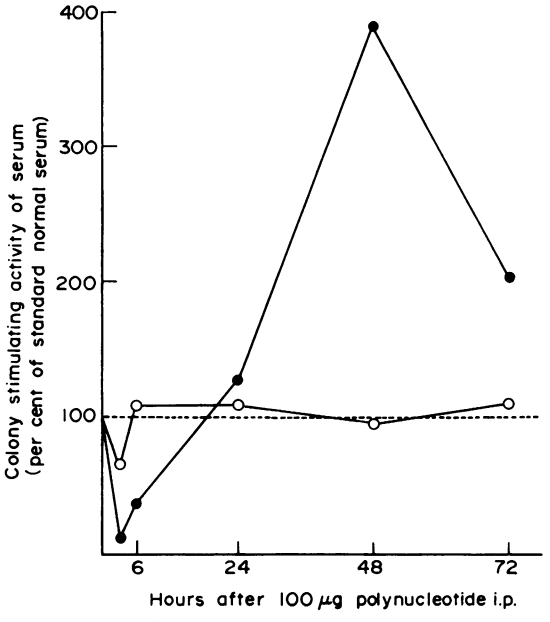

FIG. 2. Serum colony stimulating activity following i.p. inoculation of 100 μg (●) Poly I–Poly C, or (○) Poly AU. Expressed as a percentage of the activity of the same volume of normal serum.

expressed as a percentage of that due to the same volume of a standard normal serum pool. In response to Poly I–Poly C the colony stimulating activity fell to a low level during the period immediately following inoculation and this was followed by a rise to four times normal 48 hours after inoculation. With Poly AU there was a slight early fall and no subsequent increase above normal levels.

The fall in colony stimulating activity in response to Poly I–Poly C was unexpected and was investigated in more detail. The first question was whether the fall was due to loss of colony stimulating factor or to the presence of a colony inhibitor. This question was resolved by adding 3 hour Poly I–Poly C serum to cultures which also contained colony stimulating factor provided by conditioned medium (Bradley and Sumner 1968), and comparing colony development in these cultures with that in cultures containing either conditioned medium alone or Poly I–Poly C serum alone. Table 2 shows that the low colony stimulating activity of the Poly I–Poly C serum was due to inhibition of colony development. This inhibition was not due to some non-specific toxicity since the serum

TABLE 2

INHIBITORY EFFECT OF 3 HOUR POLY I–POLY C SERUM ON CONDITIONED MEDIUM STIMULATED CULTURES OF NORMAL BONE MARROW

Stimulus	Colonies per culture (AV of 3)
Conditioned medium	63
3 HR Poly I–Poly C serum	2
Conditioned medium + 3 HR Poly I–Poly C serum	2

which was inhibitory for mouse colonies had no effect on cultures of normal hamster bone marrow as shown in Table 3. Hamster marrow was cultured in exactly the same way as that from mice.

It has been shown that the number of colonies developing from a constant number of marrow cells in a given set of cultures is proportional to the amount of colony stimulating

TABLE 3

EFFECT OF 3 HOUR POLY I–POLY C MOUSE SERUM ON CULTURES OF HAMSTER BONE MARROW

Stimulus	Colonies per culture (AV of 4 ± SD)
Normal hamster + normal mouse serum	157 ± 20
Normal hamster + 3 HR Poly I–Poly C mouse serum	150 ± 14

factor added to the cultures (Bradley, Metcalf, Sumner and Stanley, 1969). It was of interest therefore to compare the colony response to a series of doses of the inhibitory serum with that to the same doses of normal serum. This comparison showed (Fig. 3) that the inhibitory effect of the three hour Poly I–Poly C serum only became apparent in the higher range of doses and that at low doses it gave greater stimulation than normal serum.

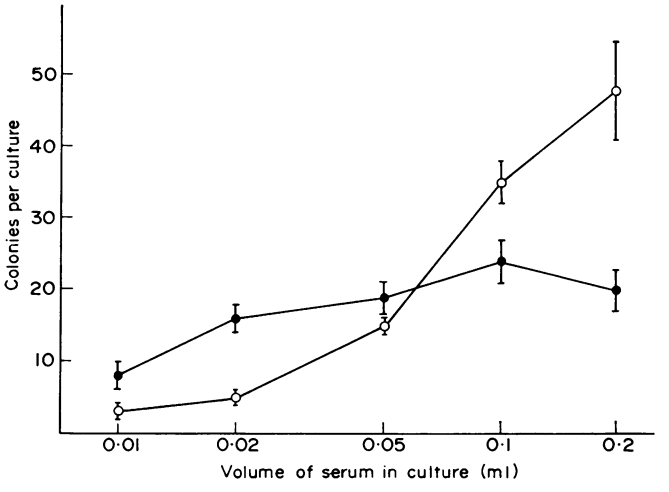

FIG. 3. Relationship between colony number and amount of serum used to stimulate cultures. ○, Normal serum; ●, serum from mice 3 hours after 100 μg Poly I–Poly C i.p. Bars represent ± 1 SD of mean colony number.

An important consequence of this 'cross-over' effect is that estimates of serum colony stimulating activity based on the effect of a single concentration of serum could be misleading and any inhibitory activity could be overlooked if a sufficiently high dose of serum is not used. Fig. 4 shows the colony stimulating activity of the Poly I–Poly C serum pools depicted in Fig. 2 expressed in the same way but using two different doses of serum. It is obvious that the profile of the response was very dependent upon the dose of serum used

for assay and that with the lower dose no inhibitory effect was apparent. Similar comparisons of colony stimulating activity with two doses of serum were made for serum pools from groups of five mice bled at intervals after injection of 5 μg bacterial endotoxin and 0·2 ml Freund's complete adjuvant. The serum colony stimulating factor response to these agents also shown in Fig. 4 had the same profile with each dose of serum but it is interesting that with endotoxin the lower dose of serum gave higher levels of colony stimulation relative to normal serum whereas with Freund's complete adjuvant the opposite effect was seen.

Perhaps the best known biological property of Poly I–Poly C is its effectiveness as an inducer of interferon both *in vivo* and *in vitro*. In the light of the colony inhibiting effect of serum taken shortly after inoculation of Poly I–Poly C it seemed obvious to investigate

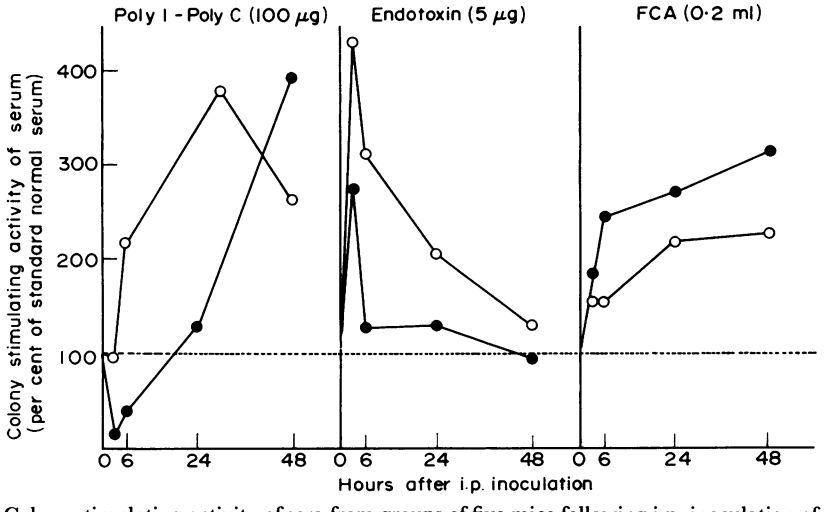

FIG. 4. Colony stimulating activity of sera from groups of five mice following i.p. inoculation of 100 μg Poly I–Poly C, 5 μg shigella endotoxin, and 0·2 ml Freund's complete adjuvant. Expressed as a percentage of the activity of normal serum and tested at (●) 0·05 ml per culture, and (○) 0·02 ml per culture.

the relationship between colony inhibitor and interferon. The first step in this investigation is shown in Fig. 5 where colony stimulating activity and interferon activity of serum pools taken 3, 6, 24 and 48 hours after each of three intraperitoneal inoculations of 100 μg Poly I–Poly C are shown. This indicates that the colony inhibition effect is at least temporally related to the interferon responses. Fig. 6 shows the interferon and colony stimulating activity of the endotoxin and Freund's adjuvant serum pools depicted in Fig. 4, and also these activities following intraperitoneal inoculation of 800 HAU of influenza virus. This shows that a high titre of interferon was again associated with colony inhibition, that a low titre of interferon following endotoxin while not associated with obvious colony inhibition was associated with a disproportionally low level of stimulating activity with increasing dose (Fig. 4), and that the sera following Freund's adjuvant which did not show this effect did not contain any detectable interferon.

DISCUSSION

A comparison of the spleen CFC response to intraperitoneal inoculation of 50 μg of

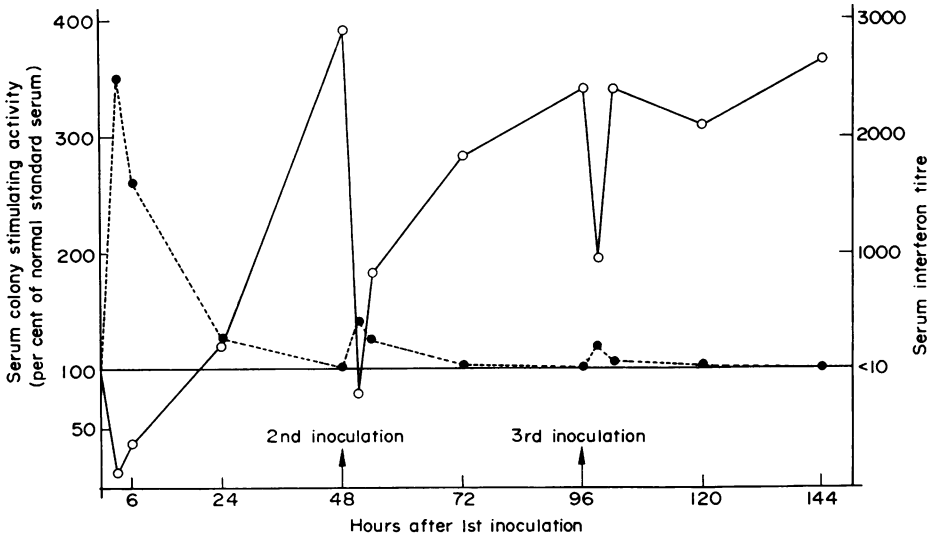

FIG. 5. Colony stimulating and interferon activity of serum pools from groups of five mice following three i.p. inoculations of 100 μg Poly I–Poly C at 48 hour intervals. ○, Colony stimulating activity; ●, interferon activity.

either Poly I–Poly C or Poly AU showed that Poly I–Poly C had a weak stimulatory effect and that Poly AU had a negligible effect. The response to 50 μg Poly I–Poly C was of the same magnitude as that shown previously with 1 μg of flagellin (McNeill, 1970c). Since Poly AU gave a better colony potentiation *in vitro* (McNeill, 1971) it can be concluded that the colony potentiation phenomenon plays very little part in the CFC response to injected materials *in vivo*. Previous work has shown that the CFC response *in vivo* is associated with an increase in the level of colony stimulating factor in the serum (McNeill, 1970c). The present experiments show that serum colony stimulating factor

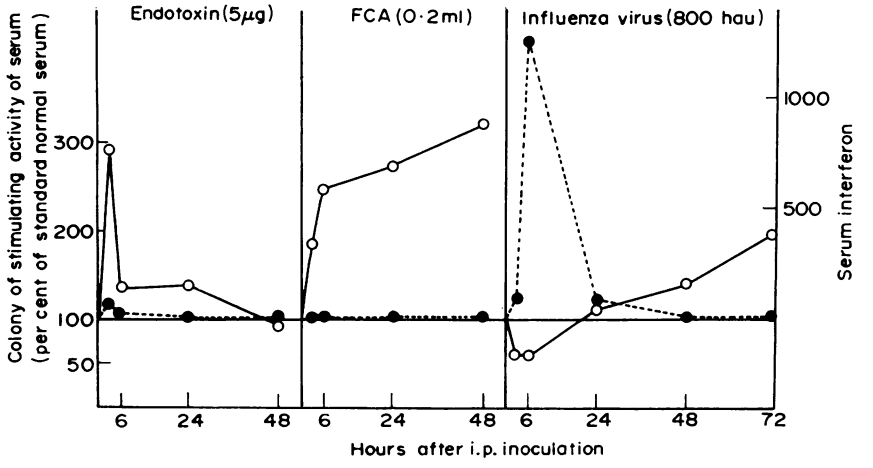

FIG. 6. Colony stimulating and interferon activity of serum pools following i.p. inoculation of Shigella endotoxin, Freund's complete adjuvant, and influenza virus. ○, Colony stimulating activity; ●, interferon activity.

rises in response to Poly I–Poly C but not in response to Poly AU and it therefore seems likely that the CFC response *in vivo* is largely dependent upon release of colony stimulating factor, particularly since the rise in stimulating factor occurs before the increase in spleen CFC. It is interesting that Poly I–Poly C and Poly AU which are structurally similar can differ so much in their effects on the CFC system *in vitro* and *in vivo*. Evidence has been presented which suggests that colony potentiation *in vitro* is dependent upon an action at the cell surface (McNeill, 1971). If cellular uptake was required for release of colony stimulating factor the difference between the two compounds could be simply explained by differences in their rate of uptake, i.e. if Poly AU was taken into cells less readily than Poly I–Poly C it would be more effective in colony potentiation but less effective in causing release of colony stimulating factor and a consequent CFC response *in vivo*.

Previous experiments on CFC responses *in vivo* showed that there was a detectable increase in the number of spleen CFC by 24 hours after inoculation of a variety of antigens and adjuvants (McNeill, 1970c), and the present experiments show that the colony stimulating factor responses to two of these agents are present 3 hours after inoculation (Fig. 4). With Poly I–Poly C, however, the CFC response was not detected until the third day after inoculation and this delay is probably related to the later colony stimulating factor response. The colony stimulating factor response to Poly I–Poly C was complicated by the fact that its profile depended upon the dose of serum used for assay. With higher doses the 3 and 6 hour sera actually showed a fall in colony stimulating activity. This was clearly due to the presence of an inhibitor of colony growth rather than to a loss of colony stimulating factor since the serum was shown to inhibit the activity of conditioned medium. The dependence of the colony stimulating profile on the dose of serum used for assay could be explained if (a) the sera contained both colony stimulating factor and inhibitor and (b) if the colony stimulating factor had a lower threshold for activity than the inhibitor. Under such circumstances the resultant effect of stimulation and inhibition would favour inhibition at higher serum doses and stimulation at lower doses. When 3 hour Poly I–Poly C serum was compared with normal serum over a range of doses from 0·01 ml to 0·2 ml per culture (Fig. 3), it gave a greater degree of stimulation at 0·01 ml and 0·02 ml and less stimulation than the normal serum at 0·1 ml and 0·2 ml per culture. The increased stimulatory activity with low serum doses is not necessarily due to an increase in serum colony stimulating factor since Poly I–Poly C still present in the serum may have resulted in a colony potentiation effect.

Although further characterisation of the colony inhibitor will require separation from colony stimulating factor several other points can be made. First, the 'cross-over' effect of inhibitor-containing serum when compared with normal serum could be used as an indication of the presence of inhibitor in whole serum. Fig. 4 shows this very clearly with Poly I–Poly C sera and also to a lesser extent with sera obtained after injection of endotoxin. In the case of endotoxin although there was a marked increase in serum colony stimulating activity during the 6 hours after inoculation the effect was greater when the sera were assayed at 0·02 ml per culture than when a dose of 0·05 ml was used. In contrast, the response to Freund's complete adjuvant relative to normal serum was greater when assayed at the higher dose. On the basis of the foregoing interpretation these experiments suggest that a small amount of inhibitor appears in response to endotoxin but not in response to Freund's adjuvant.

The second point is that the inhibitory effect is specific since a concentration of serum

which inhibited growth of mouse bone marrow colonies had no effect on the growth of colonies from hamster marrow. This observation also makes it unlikely that inhibition in the mouse system was due to injected materials which may have been present in the blood during the period immediately following inoculation.

Finally, since it is well known that Poly I–Poly C induces a high level of serum interferon these results raise the possibility that colony inhibition is mediated by interferon. The data shown in Fig. 5 indicate that there is a close temporal relationship between serum interferon responses and the decrease in colony stimulating activity following each of three intraperitoneal inoculations of Poly I–Poly C. The possibility that colony inhibitor and interferon are closely related is further emphasized since it was suggested above that a small amount of inhibitor appeared in response to endotoxin but not in response to Freund's adjuvant, and these were precisely the interferon responses observed (Fig. 6). In addition, a high interferon response to influenza virus was associated with marked colony inhibition. In view of the occurrence of both colony inhibitor and colony stimulating factor in the same sera separation of these activities is essential in order to clarify the relationship between them. Such experiments will be reported in a subsequent paper.

ACKNOWLEDGMENT

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