Interaction of Toxic Lectin Ricin with Epithelial Cells of Rat Small Intestine *in Vitro*[1)]

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To clarify the mechanism of oral toxicity of ricin, the interaction of ricin with the epithelial cells isolated from rat small intestine was compared *in vitro* with those of other plant lectins by two different determinations, *i.e.*, viability and cytotoxicity. After incubation of the cells for 1 h at 37 °C with ricin, ricin B-chain, castor bean hemagglutinin (CBH), soybean agglutinin (SBA), wheat germ agglutinin (WGA), concanvalin A (Con A), and peanut agglutinin (PNA), respectively, followed by staining with trypan blue, ricin and ricin B-chain as well as CBH and SBA were found to have effectively reduced the number of viable cells. On the contrary, only ricin inhibited protein synthesis in the cells and the effect was blocked by D-galactose. Additional experiments employing [^{125}I]-labeled ricin strongly suggested that ricin was first bound *via* its B-chain to the galactosyl residues on the cell surface followed by internalization into cells as the whole 62 kDa molecule.

These results infer first that ricin, as well as other lectins mentioned above, was able to reduce viability of the epithelial cells of rat small intestine by direct binding to the cell surface. The second effect, specific to ricin, was the inhibition of cellular protein synthesis.

Keywords ricin; lectin; phytohemagglutinin; rat small intestine; oral toxicity; epithelial cell; viability

Ricin D, a toxic lectin (62 kDa) in castor bean seeds (*Ricinus communis* L.), has been shown to consist of two polypeptide chains (A- and B-chains), which are linked together by a single disulfide bond.[2)] The binding of its B-chain (32 kDa) to the cell surface glycoconjugates having non-reducing terminal galactose allows the penetration of ricin into the cell, followed by the release of the A-chain (30 kDa), which then inhibits the cellular protein synthesis in eucaryotic cells, resulting in this lectin's cytotoxic effect.[3)] In previous papers, we have shown that ricin exerts its toxic action after oral administration even though it is a proteinous substance which can be degraded in the stomach and intestines.[1,4)] It was also inferred that ricin administered *p.o.* acted primarily on the intestinal mucosa and impaired sugar absorption of the small intestine.[4a)] Ricin-B chain and ricin are both cytotoxic to the epithelial absorptive cells of the small intestine, but impairment of sugar absorption by the small intestine alone is not the direct cause of death of animals following oral administration of ricin.[1)] Also revealed by light microscopic examination were significant changes such as loss of villi and delay of redegeneration of the absorptive epithelials of the small intestine soon after administration.[4b)] However, whether or not only ricin causes such significant morphological changes to the intestinal mucosa remains unsolved. There have been no papers concerning the effect of ricin on the normal epithelial cells of small intestine *in vitro*, although numerous studies have employed transformed or tumor cells for the elucidation of toxic action of ricin *in vitro*.[3a,5)] In the present paper, the ricin effects on the dispersed epithelial cells of rat small intestine were compared with several other plant lectins by two different methods to clarify the relationship between the significant changes in the intestinal mucosa and ricin's oral toxicity. The results demonstrated that ricin had deleterious effects *in vitro* on rat small intestinal cells in two different ways, one was its direct interaction with the cell membrane and the other its inhibition of protein synthesis after internalization.

Materials and Methods

Ricin D and castor bean hemagglutinin (CBH) were prepared according to the procedure reported by Hara *et al.*[6)] and its two polypeptide chains were obtained by reducing a single disulfide bond with 2-mercaptoethanol (2-ME) in the presence of 0.1 M D-galactose at room temperature overnight, followed by diethylaminoethyl (DEAE)- and carboxymethyl (CM)-cellulose column chromatographies.[2,7)] The purity of each chain isolated was judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Wistar-strain rats, weighing 180—220 g, and laboratory chow (Oriental MF) were purchased from Otsubo Experimental Animals, Nagasaki. Neomycin, nystatin and bovine serum albumin (BSA) were obtained from Sigma; streptomycin and penicillin G from Meiji; Eagle's minimum essential medium (MEM) from Nissui. [^{125}I]sodium and [^{3}H]leucine were purchased from the New England Nuclear (NEN). Lectins [concanavalin A (Con A), peanut agglutinin (PNA), soybean agglutinin (SBA), and wheat germ agglutinin (WGA)] were kindly donated by Dr. T. Yamamoto, Seikagaku Kogyo Co., Tokyo and Dispase by Dr. J. Yoshitake, Godo Shusei Co., respectively. All other reagents used were of analytical grade.

Preparation of the Epithelial Cells from Rat Small Intestine[8a)] To reduce the effect of intestinal microorganisms, beginning 72 h prior to sacrifice animals were given neomycin in drinking water (10 mg/30 ml tap water). Food was withheld for 2 d before sacrifice and during the final 24 h rats were kept under complete oral restriction. At 16 h before sacrifice, each rat received 24000 U of nystatin, 500 mg of streptomycin, and 200000 U of penicillin G.[8b)] Rats were killed by decapitation and the small intestine was removed, divided into six parts, washed with 0.9% sodium chloride solution, everted and ligated, then filled with 0.9% sodium chloride solution to produce a slight distention. Sacs were incubated at 37 °C for 15 min in 25 ml of phosphate buffered saline solution (PBS), pH 7.4, containing 400 U/ml Dispase and 1% BSA. The incubation medium was filtered with nylon mesh and the cells were collected by centrifugation (500 × *g*, 1 min). After washing twice with the same medium, about 1—5 × 10^7 viable cells per rat, as determined by trypan blue exclusion method, were routinely obtained.

Effect of Ricin on Viable Cell Population Epithelial cells (4 × 10^5 cells/tube) were incubated with a toxin or lectins independently at 37 °C in 0.2 ml of PBS containing 1% BSA. After incubation, a portion of the mixture was taken out and stained with 0.2% trypan blue solution. The unstained (viable) cells were counted and the viable cell population (viability) was expressed as a percent ratio of viable cell numbers to the total cell numbers at a given time. Assay was performed in triplicate, and the results were expressed as the mean values from three separate experiments.

Determination of Inhibition of Cellular Protein Synthesis Cells (2 × 10^6 cells/ml) were suspended in L-leucine-free Eagle's MEM containing 5% calf serum, 10 μg/ml insulin, 4.5 mg/ml glucose, and 4 mM L-glutamine.[9)] Cells were first incubated with increasing concentrations of ricin, its A- and B-chain, or other lectins, separately, for 50 min at 37 °C in a 5% CO_2 incubator. After addition of 11.1 kBq of [^{3}H]leucine, the

mixtures were further incubated for 60 min. Proteins were precipitated by adding 4% perchloric acid (PCA). The precipitates were filtered on Whatman type GF/C glass fiber filters.[10] The filters were washed six times with 4% PCA, three times with absolute ethanol and then dried. The radioactivity on the filter was counted by a liquid scintillation counter (Aloka LSC-700) in 10 ml of Aquasol II (NEN). Each assay was carried out in triplicate.

Iodination of Ricin and Its B-Chain Ricin (1.5 mg, 23.4 nmol) and its B-chain (1.5 mg, 45 nmol) were iodinated for 10 s with 18.5 MBq of [^{125}I]sodium (NEN) using chloramine T. Iodination was stopped by adding sodium iodide and sodium thiosulfate, and iodinated proteins were separated from free reagents by passing through a Sephadex G-50 column with 5 mM phosphate buffer, pH 7.4, containing 80 mM sodium chloride and 0.1% BSA.

Competitive Binding Assay This was carried out in a system where epithelial cells (1×10^5 cells/tube) were incubated with [^{125}I]ricin (8×10^4 cpm/tube, 6×10^{-9} M) and varying concentrations of unlabeled ricin, ricin B-chain or CBH for 10 min at 4 °C. The cells were then filtered, washed and counted. Specific binding was calculated by subtracting non-specific binding, which was determined in the presence of 0.2 M D-galactose, from the total binding. To calculate the number of binding sites per cell and the apparent K_a value, the data were plotted according to Scatchard.[11] More than 90% of the bound toxin could be released upon addition of D-galactose; however, since such a small fraction was not released by this treatment at 37 °C, the binding is not truly an equilibrium phenomenon at this temperature. The binding constants were therefore obtained from data at 4 °C.

Uptake of [^{125}I]Ricin by the Epithelial Cells The cells (1×10^5 cells) were allowed to react with [^{125}I]labeled ricin (1.37 μg, 21.1 pmol) in 0.2 ml of PBS containing 1% BSA in a polypropylene tube at 37 °C or 4 °C for various periods. Cells were then washed three times with 0.6 ml of ice-cold medium containing 0.2 M D-galactose followed by centrifugation ($500 \times g$, 5 min). The remaining radioactivity in the pellet was determined as a measure of the irreversible binding, due to its internalization into the cells. After 200 μl of diluted PBS containing 1% BSA was added to the cell pellet, the mixture was sonicated for 5 s, then centrifuged at $15000 \times g$ for 10 min. The supernatant solution was freeze-dried, resuspended in 20 μl of 32 mM Tris–HCl buffer (pH 6.8) containing 1% SDS, 5% glycerol, and 0.001% bromphenol blue, and analyzed by SDS-PAGE (12.5% gel) and autoradiography.[12]

Results

Cytotoxicity of Ricin and Other Lectins towards the Intestinal Epithelial Cells The effect of ricin and its polypeptide chains on the population of viable cells was studied by the trypan blue exclusion method. Over 90% of the epithelial cells, isolated by the treatment with Dispase from rat small intestine, were viable in this preparation method. The viability gradually reduced to about 80% after incubation at 30 °C for 2 h and to 65% after 5 h. From this observation, all experiments were planned for completion within 2 h. It was shown that ricin and its B-chain rapidly reduced the numbers of the viable cells in parallel with prolongation of the incubation time (40% for ricin and 60% for B-chain after 30 min; 38% for ricin and 50% for B-chain at 60 min; 30% for both after 2 h), while A-chain had little effect. As shown in Fig. 1, this cytotoxic effect of ricin, when compared at 60 min of incubation, was both concentration- and temperature-dependent, and the effect was blocked by 0.1 M D-galactose. CBH and SBA showed the similar effect, whereas Con A, WGA and PNA were ineffective (Fig. 2).

Effect of Lectins on Protein Synthesis in Epithelial Cells As shown in Fig. 3, only ricin inhibited protein

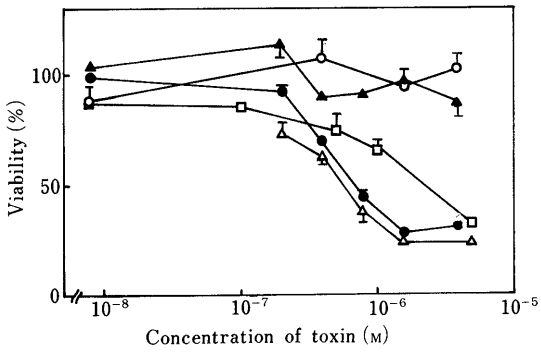

Fig. 1. Effect of Ricin and Its B-Chain on Viability of Epithelial Cells from Rat Small Intestine

Epithelial cells (4×10^5 cells/tube) were incubated at 37 °C, 15 °C and 4 °C for 60 min with an increasing amount of lectins in the presence or absence of 0.1 M D-galactose. After incubation, an equal volume of 0.2% trypan blue solution was added to each tube. The number of living (unstained) cells was counted and expressed as the percent of viability against the cell number incubated in the absence of lectins. Values represent mean ± S.D. of triplicate determinations from three separate experiments. ○, ricin at 4 °C; □, ricin at 15 °C; ●, ricin at 37 °C; △, ricin B-chain at 37 °C; ▲, ricin + D-galactose at 37 °C.

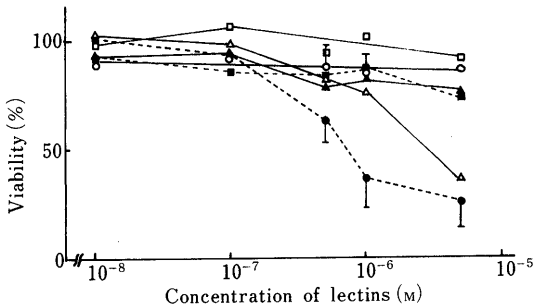

Fig. 2. Viability of Epithelial Cells after Treatment with Lectins

Cells (4×10^5 cells/tube) were incubated at 37°C for 60 min and the viability was determined as in Fig. 1. In the case of CBH, the effect of D-galactose was also included. ■, WGA; □, Con A; ▲, PNA; △, SBA; ●, CBH; ○, CBH + 0.1 M D-galactose.

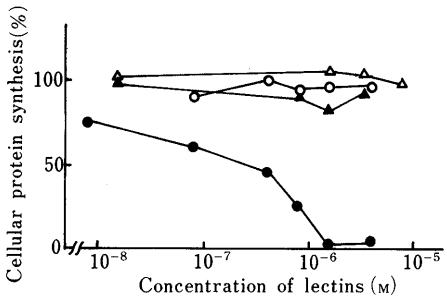

Fig. 3. Effect of Ricin and Its Constituent Chains on Protein Synthesis in Epithelial Cells from Rat Small Intestine

Cellular protein synthesis in the epithelial cells from rat small intestine was determined after pretreatment of the cells with ricin or its chains followed by further incubation with [^{3}H]leucine at 37 °C. Details are described in Materials and Methods, and each value represents the mean of triplicate determinations. ●, ricin; △, ricin A-chain; ▲, ricin B-chain; ○, ricin + 0.1 M D-galactose.

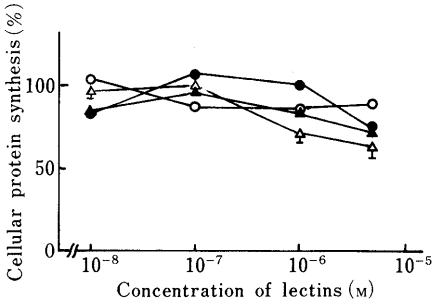

Fig. 4. Effects of Lectins on Cellular Protein Synthesis in Rat Intestinal Cells

Protein synthesis was determined in the presence of various individual lectins as described in the text. ○, PNA; ●, WGA; △, SBA; ▲, CBH.

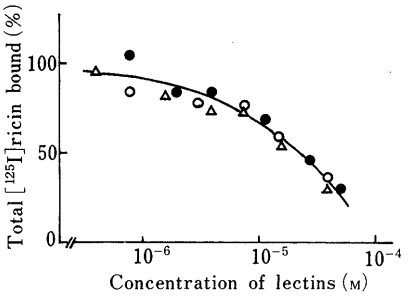

Fig. 5. The Competitive Inhibition of [^{125}I]Ricin Binding to the Rat Intestinal Cells by Unlabeled Ricin, Its B-Chain or CBH

Epithelial cells (1×10^5 cell/tube) were incubated for 10 min at 4 °C with [^{125}I]ricin (8×10^4 cpm or 6×10^{-9} M) and varying concentrations of unlabeled lectins to give the indicated total concentrations. Radioactivity was determined after washing and centrifugation as described in the text. △, ricin; ○, ricin B-chain; ●, CBH.

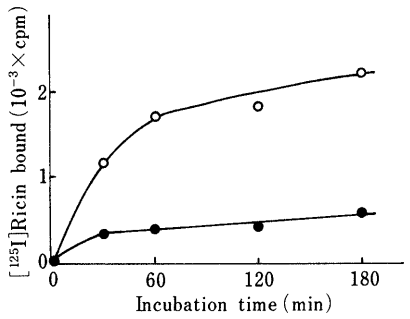

Fig. 6. Irreversible Binding of [^{125}I]Ricin to Epithelial Cells from Rat Small Intestine

Cells were incubated at 37 °C or 4 °C for the indicated periods of time with [^{125}I]ricin (1×10^5 cpm or 8×10^{-9} M). The cells were washed exhaustively with PBS containing 0.2 M D-galactose and 1% BSA followed by centrifugation ($500 \times g$, 5 min). The radioactivity of the pellet was counted as the irreversible binding due to internalization. ○, 37 °C; ●, 4 °C.

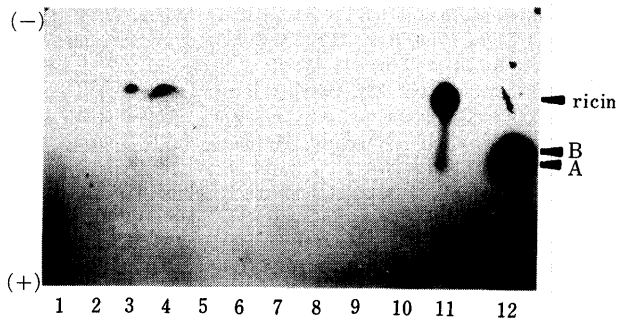

Fig. 7. Internalization of [^{125}I]Ricin by Epithelial Cells

Cells were incubated with [^{125}I]ricin at 37 °C or 4 °C for the indicated periods of time and then after washing the irreversible binding was determined as in Fig. 6. The cell pellets obtained were suspended in PBS, sonicated and centrifuged. The supernatant solution was analyzed by SDS–PAGE and autoradiography as described in the text. Lane 1—5, incubated at 37 °C; lane 6—10, incubated at 4 °C; lane 1, 6, 0 min; lane 2, 7, 30 min; lane 3, 8, 60 min; lane 4, 9, 120 min; lane 5, 10, 180 min; lane 11, [^{125}I]ricin without 2-ME; lane 12, reduced [^{125}I]ricin (+2-ME).

synthesis in the cells and this inhibition was blocked in the presence of 0.1 M D-galactose. It was obvious that neither A- nor B-chain alone was responsible for the inhibition. Other lectins such as PNA, WGA and CBH did not exhibit inhibitory action on cellular protein synthesis over a wide range of concentrations between 1×10^{-8} and 5×10^{-6} M although SBA exhibited slight inhibition at 5×10^{-6} M (Fig. 4).

Binding of Ricin to Rat Intestinal Cells Preliminary binding experiments employing [^{125}I]ricin (9.4×10^4 cpm/μg or 4.6×10^4 cpm/tube) and the epithelial cells (1×10^5 cells/tube) were carried out according to the method described by Sandvig *et al.*[13]; the binding of [^{125}I]ricin proceeded rapidly during the initial 10 min and reached a plateau in 60 min at 4 °C, as well as at 37 °C. Similar results were obtained with [^{125}I]ricin B-chain. With respect to the effect of temperature on the binding, the amounts of [^{125}I]lectins bound to the cells at 4 °C were evidently greater than those at 37 °C (data not shown). This binding was inhibited completely at 4 °C in the presence of 0.2 M D-galactose, although at 37 °C nearly 10% of the total activity bound to the cell could not be removed despite exhaustive washing with D-galactose.

The binding nature of ricin to rat intestinal cells was studied by competitive binding assay as shown in Fig. 5, and [^{125}I]ricin was demonstrated to be competitively inhibited by either unlabeled ricin, ricin B-chain or the homologous lectin CBH. Scatchard analysis of the data suggested the presence of binding sites (1.5×10^7 sites/cell) with K_a of 3×10^6 M^{-1}. Figure 6 shows the irreversible binding of [^{125}I]ricin to the cells at 4 °C and 37 °C, and suggests that a fraction of [^{125}I]ricin may be internalized into cells at the higher temperature. Figure 7 shows an autoradiography of SDS-PAGE analysis of the cytosolic fraction. The radioactivity was mainly found at the position of whole ricin (62 kDa) and very weakly at the positions of individual subunits (about 30 kDa); density of bands increased with incubation time at 37 °C, but little was observed at 4 °C. It was concluded that the radioactivity estimated as irreversible binding was derived primarily from the labeled whole ricin molecule and only partly from the polypeptide chains probably cleaved at an interchain disulfide bond, which was, after binding to the cells, internalized into the cells at 37 °C.

Discussion

In 1971, employing Ehrlich ascites tumor cells and Yoshida ascites hepatoma cells, Lin *et al.* first demonstrated that the cellular protein synthesis was inhibited by ricin within 60 min, followed by later inhibition of deoxyribonucleic acid (DNA) synthesis, whereas no appreciable inhibition of ribonucleic acid (RNA) synthesis occurred.[3a] They also reported that cell death was recognized long after the inhibition of protein synthesis. Their results inferred that ricin is more effective to tumor cells than to the normal cells, and this lectin has since commanded considerable attention because of its application in the preparation of immunotoxins, where tumor cell-specific antibodies are used to direct the toxin or its enzymatically active A-chain towards specific target cells.[3f] Olsnes and his group later confirmed these results and studied in further detail the binding of ricin, its internalization and translocation, and inhibition of protein synthesis in HeLa cells.[3c,d] However, very few studies have appeared on the *in vitro* toxic effects on normal cells because of technical difficulties in culturing these cells, although ricin-conjugate such as ricin–horseradish peroxidase was shown immunohistochemically to bind certain normal cells *in vivo*.[3e]

Ricin has long been known to be lethally toxic to animals regardless of the route of administration, s.c., i.p., or i.v.[14a,b] One characteristics is that when orally given and after a lag period the lectin can elicit symptoms like heavy diarrhea

and hypothermia, followed by death of the animal.[4a,b] In addition, much interest has recently centered on the role of plant lectins as a foodstuff and a number of papers have reported that certain seed proteins including lectins can cause heavy gastrointestinal damage.[15a-f] It thus seems very important to explore the *in vitro* effect of ricin on normal epithelial cells isolated from rat intestinal mucosa, and to investigate the mechanism of oral toxicity of ricin in rats.

As shown in the present study, ricin's cytotoxic effects on epithelial cells can be explained by two different processes: one is a direct interaction with the cell membrane detected by trypan blue exclusion method, and the other inhibition of the protein synthesis in the cells. The former may be due to direct binding of ricin to the membrane through galactosyl residues of the cell surface glycoconjugates, since this effect was found temperature-dependent as well as D-galactose-sensitive (Fig. 2). It was inferred that ricin, its B-chain and CBH had the same or similar binding sites on the epithelial cells (Fig. 5).

There have been many studies on the biological function of plant lectins.[16a-h] Phytohemagglutinins in general possess mitotic stimulating activity[16f]; Con A exerts its carcinostatic effect by binding to the membrane but lacks the ability to inhibit cellular protein synthesis[16g]; ricin, its B-chain and CBH increased membrane fluidity of human peripheral lymphocytes and CBH had a mitogenic activity in these cells, although whether or not it inhibited protein synthesis was not determined.[16h] These results suggest that lectins, in general, affect membrane fluidity and cell division, sometimes resulting in the death of cells. The cytotoxicity of ricin and its B-chain demonstrated by trypan blue staining appears due to these effects on the epithelial cell membrane of rat small intestine.

The second specific effect of ricin on the epithelial cells was the inhibition of cellular protein synthesis, although B-chain, CBH, and SBA were ineffective (Fig. 3). To explain this specific inhibition, the binding and internalization of [^{125}I]ricin was explored. By binding experiments in the epithelial cells, it was inferred that the sensitivity of these cells of the rat small intestine is almost equal to that of the transformed or tumor cells,[13,17] although strict comparison is not possible due to the differences in experimental conditions. This binding of ricin was D-galactose-sensitive and its constituent B-chain was responsible for the binding capacity (Fig. 5). The irreversible binding of [^{125}I]ricin, after intensive washing with D-galactose solution, indicates the internalization of ricin into the epithelial cells. Internalization of the whole ricin (62 kDa) was further confirmed by SDS-PAGE and autoradiography (Figs. 6 and 7), as well as by the presence of a very trace amount of low molecular chains (−30 kDa) (Fig. 7, lanes 3 and 4). A similar process of internalization was reported in HeLa cells,[3d,g] in which, after ricin binding and internalization, the A-chain, which is somehow released, translocates across the membrane of an endocytic vesicle in the cytoplasm where it catalytically inactivates 60S ribosomal subunits, thereby inhibiting protein synthesis and causing cell death.

The results obtained in the present study suggest that the epithelial cells of rat small intestine are sensitive to ricin, and, as in HeLa cells, that the lectin first interacts with the cell surface, binds to galactosyl residues, disturbs membrane structure by its B-chain, then is internalized into cells, and inhibits the protein synthesis by the released A-chain, and finally induces death of the cells. Accompanying the death of the epithelial cells is an acceleration the delay of redegeneration of intestinal villi, and this leads finally to the severe morphological changes in the gastrointestinal tract that are usually observed in the oral intoxication of ricin.

These results, however, cannot explain animal death, although these effects may cause the severe diarrhea usually observed in ricin-intoxicated animals. As stated earlier,[4a] a trace amount of intact ricin, about 0.01% of that administered orally was entered the circulation by an unknown route, and this may accumulate in the essential organs such as liver or spleen resulting in the death of an animal. The absorption and distribution of ricin given *p.o.* are now being studied and will be published elsewhere.

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