

The Plasma Effects on LTB<sub>4</sub> Secretion

An Honors Thesis (ID 499)

by

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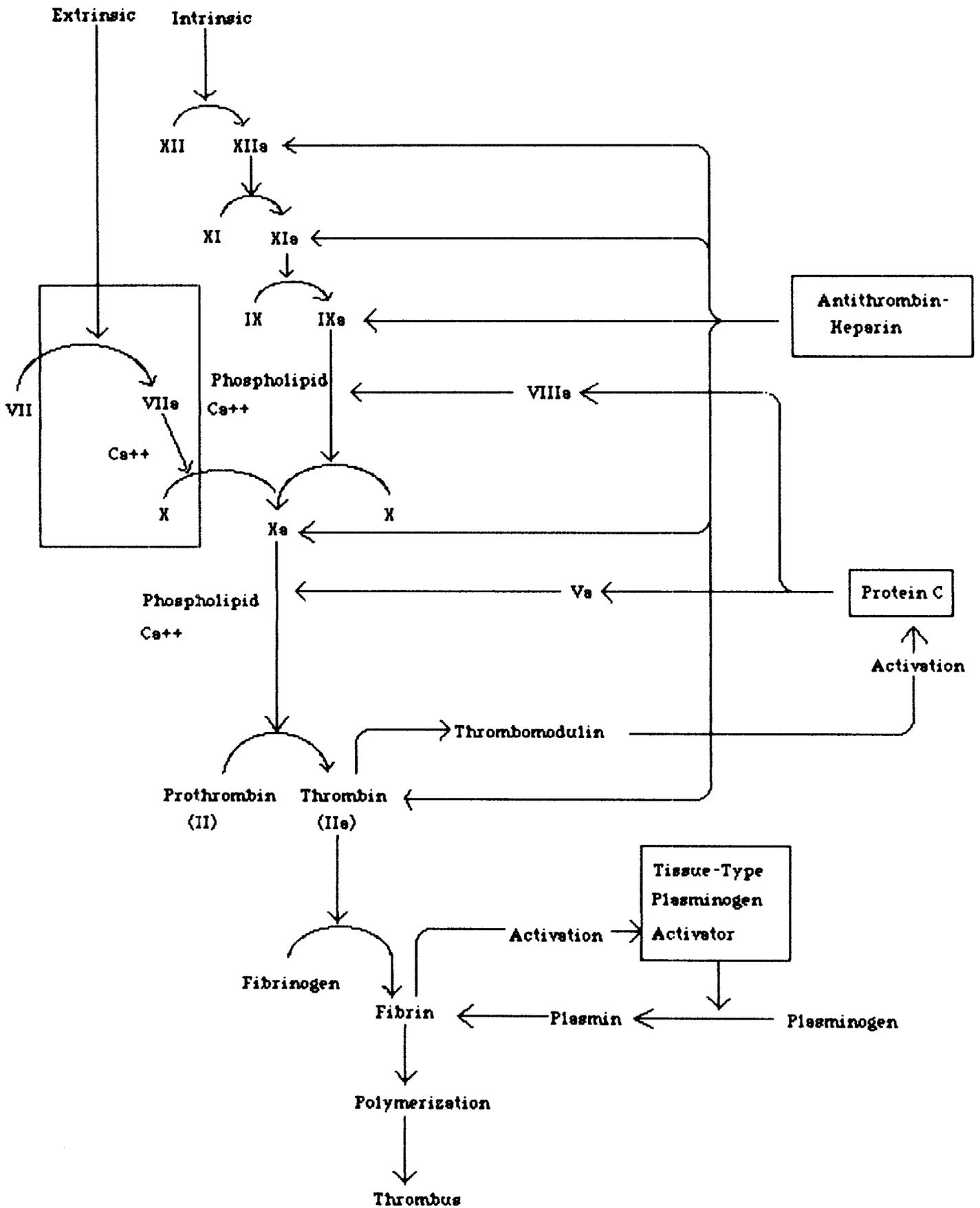
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## Introduction:

Lupus erythematosus (LE) was named in 1851 by Louis Alphee Cazenave, a French dermatologist. The name meaning "red wolf" was used to describe the wolf-bite pattern of skin discoloration associated with discoid lupus (13). There are two types of LE: discoid and systemic. Discoid lupus involves only the skin, whereas systemic can affect any body part (13). Lupus erythematosus is an age old disease; Hippocrates (5<sup>th</sup> century B.C.) described the disease in his writings, Kaposi (1872) described cases involving body parts other than the skin, and Dr. Malcolm Hargraves of the Mayo Clinic (1948) described LE cells found in the plasma of some systemic lupus erythematosus (SLE) patients (13). In 1952 Conley and Hartmann described a coagulation inhibitor in two patients with SLE (2) which seemed to interfere with the conversion of prothrombin to thrombin (see fig. 1) The inhibitor tended to be associated with SLE and for this reason the term lupus anticoagulant (LA) was used to describe the inhibitor. LA can be thought of as "immunoglobulins which interfere to a variable extent with phospholipid-dependent coagulation tests without inhibiting the activity of specific coagulation factors. (13)"

LA tend to be associated with autoimmune disorders (among them SLE) and with prolonged use of such drugs as hydralazine hydrochloride, phenothiazines, procainamide hydrochloride and quinine; LA are most often associated with the drug chlorpromazine (1,3,4,19). Some patients may develop a transient form of LA after contracting a viral infection or in response to antibiotic therapy.(14,15) There appear to be two markers which indicate the presence of LA in vivo. These markers are 1) the effects that LA has on coagulation tests and 2) LA's association with hypoprothrombinemia.

The plasma from patients with LA tends to exhibit prolonged clotting times in each of several standard phospholipid-dependent coagulation tests such as prothrombin time (PT), partial thromboplastin time



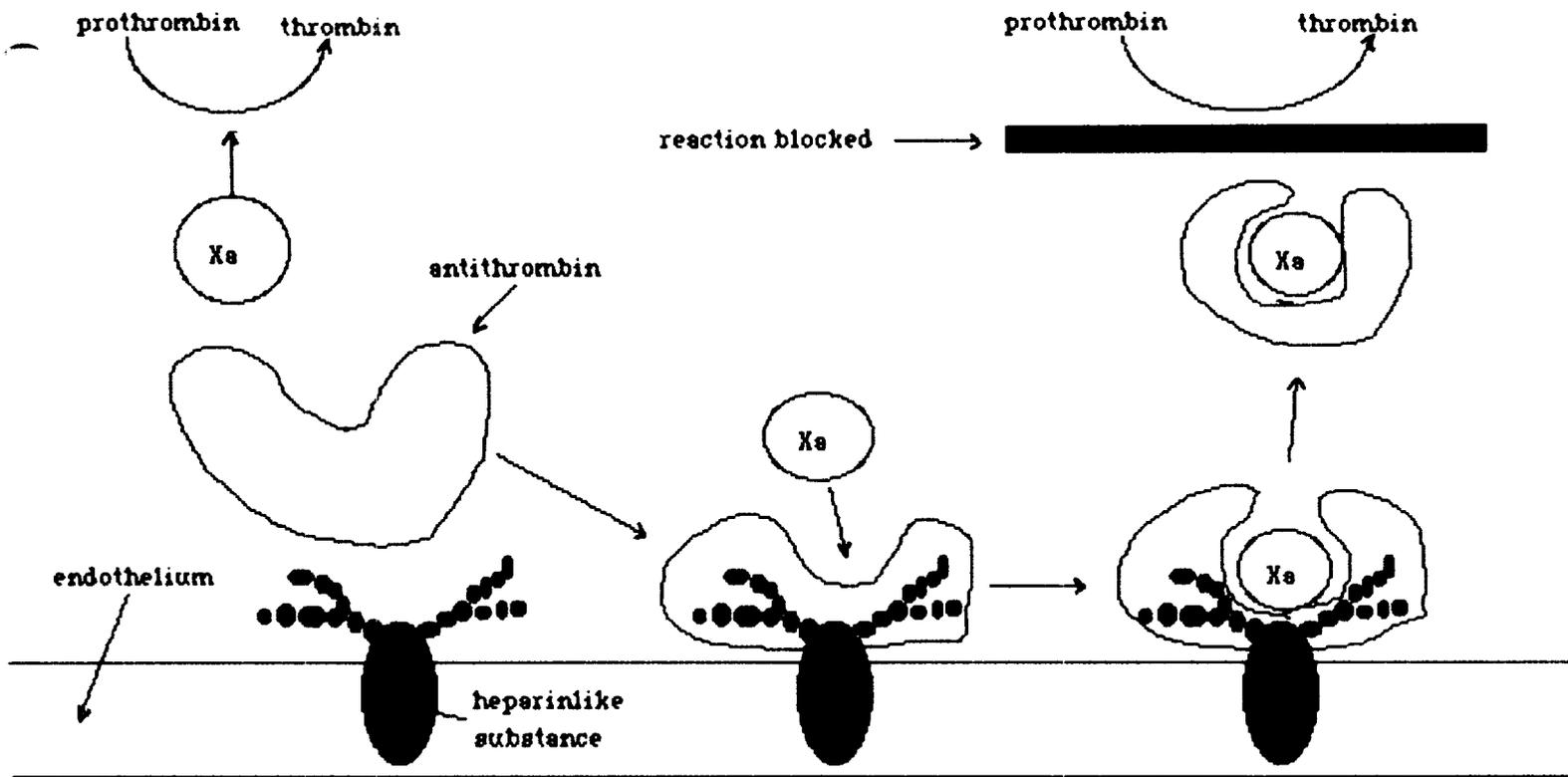
(PTT), and Russell's Viper Venom time (RVVT) (2,7,12,20). The prolonged in vitro coagulation times imply that the patients with LA are hemophiloid, however the opposite tends to be true. Most often, the occurrence of LA in patients is associated with thromboembolic episodes, primarily in the deep veins (eg. leg and cerebral veins) and peripheral arteries (10,19). To gain insight into the effects of LA on coagulation tests one needs an understanding of the coagulation cascade.

The coagulation cascade, commonly known as clotting, is a series of reactions utilizing some of the proteins carried within the plasma portion of the blood. The coagulation cascade is set into motion when a triggering agent is introduced into the vascular tree. In the patient with LA, a thromboembolic condition tends to be the norm. Thromboembolism is the blockage of a vessel with a thrombus which was formed elsewhere in the body. The standard in vitro coagulation tests are unable to detect this tendency toward thromboembolism. The tests are designed to measure only the positive side of the coagulation cascade, i.e., they are not designed to detect aspects of the regulation of the cascade (17).

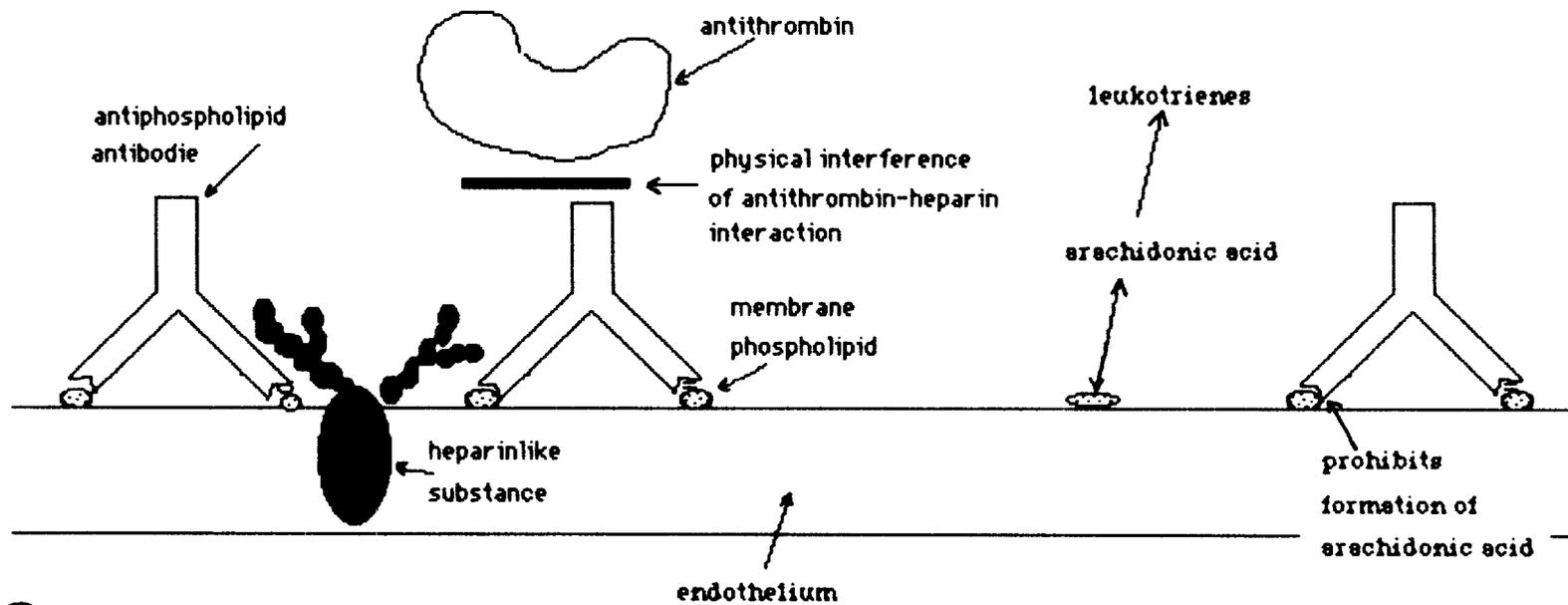
As with most systems in the body the coagulation cascade is regulated. Three mechanisms regulate the cascade through inhibition; the three mechanisms are the production of: 1) antithrombin, 2) protein C, and 3) tissue-type plasminogen activator (see fig. 1) (17). Collectively these three mechanisms perform the function of anticoagulation (regulation). The anticoagulation mechanism is mediated by these fluid phase substances found in the plasma (17). First, antithrombin which is at the center of the anticoagulation mechanism. Antithrombin neutralizes activated factors IXa, Xa, XIa, and XIIa and inhibits the activity of thrombin through the formation of protein C (17). Thrombin is the activated form of prothrombin; it causes the conversion of fibrinogen to fibrin, and fibrin is then polymerized to form an insoluble clot to slow or halt the flow of blood to an area. Next to consider is protein C which is a

stable enzyme-inhibitor which inactivates activated factors **V and VII** (17). Finally, tissue-type plasminogen activator, the last of the regulating plasma proteins to be considered. Tissue-type plasminogen activator drives fibrinolysis (the breakdown of polymerized fibrin) by transforming plasminogen to plasmin which inactivates fibrin (see fig.1) (17). The mediating fluid phase substances are intimately involved with the vascular endothelium and perform the same function as heparin (17). Heparin (an anticoagulant) catalyzes the action of antithrombin by altering the combining sites for the factors it inactivates (17). The alteration causes the reaction sites to be more accessible and therefore increases the rate of inactivation or anticoagulation (see fig.) (17). The natural heparin-like substances are partially embedded in the endothelium with the antithrombin combining sites extending into the vessel (see fig. 2) (17). These substances are present throughout the body, but predominate in the microvasculature (17). A deficiency in antithrombin leads to thromboembolic disease (a congenital disease), the spontaneous production of thrombi with the possible occlusion of a vessel. These occlusions can cause varying problems (eg. strokes, gangrene, spontaneous abortions, etc.) which cannot accurately be predicted.

Of all the documented thromboembolic disorders collected by Rosenbeg, approximately 55% had at least one thromboembolic episode and 60% of those have had recurrent episodes (17). The first episode was usually associated with an event known to trigger thrombosis such as surgery, pregnancy, childbirth or trauma (17). A deficiency of antithrombin may also be an aspect of SLE and/or the presence of LA. The occurrence of recurrent abortions in females with SLE is 3 to 4 times greater than that of a comparable population (19). The fetal wastage may or may not be due to the presence of LA in SLE patients. However, one should note that thromboembolic episodes have been shown to occur in 23-57% of patients having LA with or without SLE. In contrast,



Possible Mechanisms of APA interference with anticoagulation mechanism



thromboembolic episodes occur in 8-12% of patients afflicted with SLE and no LA (19). The presence of LA could conceivably be the cause of low antithrombin concentrations through antiphospholipid interference or some other unknown method. Antibody interference could occur via the action of antibody blockage or interference with the reaction of the heparin-like substances with antiprothrombin. If the antiprothrombin-heparin reaction does not occur, then the rate of anticoagulation will be decreased (17). Conversely, the rate of coagulation will be increased somewhat. This may not cause immediate problems, but the chance for spontaneous thrombus formation will be increased. In any event, the presence of LA is a risk factor to the occurrence of a thromboembolic episode.

Hypoprothrombinemia also appears to have some relationship to LA, but that relationship is unclear. Shapiro and Thiagarajan have shown that mild degrees of hypoprothrombinemia appear to be fairly common (27%) in their study population (19). Explanations and postulations on the mechanism behind hypoprothrombinemia have tended to be inadequate to fully explain the phenomenon. The current favored hypothesis deals with the possibility of antibody combining with prothrombin without inactivating the prothrombin (19). The logic behind the idea is that the zymogen formed via antibody combination would be rapidly cleared by macrophages. The other suggestion for the cause of hypoprothrombinemia in this case would be related to thromboembolic episodes. If a tendency towards thromboembolic episodes is present, it is possible that the rate of synthesis of prothrombin is less than the rate at which it is being used. The two most readily observable effects of LA in the human are thromboembolic episodes and hypoprothrombinemia.

The lupus anticoagulants also seem to have an effect on the inflammatory process prior to coagulation. The initial response to the introduction of a triggering agent into the vascular tree is the familiar

inflammatory reaction immediately surrounding the triggering site.

Inflammation occurs almost immediately and occurs well before coagulation can begin. The inflammatory response begins with the dilation of local arterioles and capillaries. As the vessels dilate, plasma egresses from the vessels. This fluid accumulates in the area causing localized edema. As the edema fluid accumulates, fibrin begins to polymerize and occludes lymphatic channels. These occlusions limit the dissemination of invading organisms. The polymorphonuclear leukocytes in the capillaries then begin to adhere to the endothelial wall to facilitate migration from the capillaries. The migration is stimulated by chemotactants secreted by cells near the irritation; one of these chemotactants is  $LTB_4$ . Once the leukocytes reach the area of irritation, they begin to engulf and degrade the irritant. As the degradation process continues, some of the degradative enzymes escape into the matrix surrounding the cells. These enzymes cause a drop in the pH of the area and induce the lysis of the leukocytes through the action of cellular proteases. The large mononuclear macrophages, which migrate slower than the leukocytes, arrive at the site of inflammation at this time. The macrophages engulf the leukocyte debris as well as the irritant. The arrival of the macrophages marks the final step in the inflammatory reaction (11).

One of the many macrophage cell responses during inflammation is the production and secretion of the leukotrienes (8,11). Leukotrienes are produced from the conversion of arachidonic acid (a polyunsaturated fatty acid derived from membrane phospholipids) via the lipoxygenase pathway. There are six leukotrienes (LT) produced via lipoxygenase action:  $LTA_4$ ,  $LTC_4$ ,  $LTB_4$ ,  $LTD_4$  and  $LTE_4$ . In this study the plasma effects of  $LTB_4$  were of main interest.  $LTB_4$  affects the adhesive properties of neutrophils to endothelial cells and the extravasation of white cells (6,9,18). These effects have been shown to be dependent on the concentration of  $LTB_4$  in

both in vitro and in vivo experiments (18). Other studies have shown that  $\text{LTB}_4$  is a potent chemotactic agent toward polymorphonuclear cells, eosinophils and monocytes; there have also been suggestions that  $\text{LTB}_4$  may have ionophoric activity and may mobilize membrane associated  $\text{Ca}^{2+}$  (18).

When the body is invaded by a microorganism, one of the responses may be inflammation of the area to isolate the microorganism to keep it from infiltrating the body. If the inflammatory process is impaired by decreased activity of  $\text{LTB}_4$ , then the microorganism may disseminate further than it would have and thus prolong the infection. Prolonged recovery from infections is associated with SLE and/or LA.

A thrombus causes a macrophage reaction similar to the one activated by microorganisms. One of the methods for removing a thrombus when it is formed and is no longer needed is to have the macrophages engulf and degrade it. When the macrophages are activated to remove a substance, they secrete  $\text{LTB}_4$ . One of the functions of  $\text{LTB}_4$  is to call other macrophages into the area to aid in the clearance of the clot. If  $\text{LTB}_4$  secretion is inhibited by the presence of LA or antiphospholipid antibodies then the clearance of the clot may be slower than normal. This could exacerbate thromboembolic episodes in the patient having LA.

This research project was designed to shed some light on the effects that LA have on the secretion of  $\text{LTB}_4$ , and with data from a concurrent study, to determine if the presence of antiphospholipid antibodies is related to the amount of  $\text{LTB}_4$  secretion.

## **Materials and Methods:**

**Animals** - The peritoneal macrophages utilized in this medical study were obtained from adult healthy BALB-C mice; the mice used were mixed sex ranging from 9 to 13 weeks of age and 20 to 25 grams in weight.

The mice were housed 2 to 8 to a cage and approximately 20 cages to a rack in a light-proof room with a light/dark cycle of 12 hours light and 12 hours dark. The mice had access to Purina rodent chow and water ad lib.

**Cells** - The purpose of this study was to examine plasma effects on macrophage LTB<sub>4</sub> secretion. The macrophages were obtained by the following procedure. The mice were sacrificed via cervical dislocation and the working field on the mice was disinfected by washing with 90% ethanol (EtOH). The peritoneum was then exposed by using skin snips and pulling the skin laterally to expose the peritoneal wall. The peritoneum was next washed 2 times with 5 ml of 1X PBS per washing and stored at 4°C. The peritoneal washings were centrifuged in a Beckman model TJ-6 refrigerated centrifuge (4°C) to pellet the cells. The pelleted cells were resuspended in 2 to 3 ml of a mixture of 1x RPMI 1640 (M199H-commercial) tissue culture media + penicillin and streptomycin (P/S). A count of viable macrophages was obtained by filling a hemacytometer with a 1:100 dilution of cell suspension in Trypan blue (10 microliters of resuspended cell solution in 90 microliters of Trypan blue). The cell suspension volume was adjusted to allow for each tissue culture well (Linbro tissue culture, 24 flat bottom wells, 1.7 x 1.6 cm) to contain greater than or equal to  $2.0 \times 10^6$  macrophages per well by the addition of 1X RPMI + P/S. The wells were filled with the calculated amount of cell

suspension and approximately 1 ml of RPMI + PS (to inhibit dehydration) prior to incubation. The cells were incubated in a Napco controlled environment at 5% CO<sub>2</sub> and 37<sup>0</sup>C for one hour to allow the cells to adhere to the well base. The tissue culture plates were then removed and the adhered cells in each well were rinsed 3 times with 1 x HEPES buffer warmed to 37<sup>0</sup>C in a water bath. The prepared cells were then used to test plasma effects on LTB<sub>4</sub> secretion of macrophages.

#### Plasma effects on LTB<sub>4</sub> secretion:

No more than three individual patient plasmas (IPP) were tested in one tissue culture plate at one time. Also, PNP samples were tested in parallel; one was maximally stimulated with calcium ionophore (A23187) and one was not stimulated with A23187. A23187 has been shown to stimulate the production of a maximal amount of LTB<sub>4</sub> from macrophages (18). Each tissue culture plate containing the adhered macrophages were again incubated in a total solution of 1 ml containing: 800 microliters of HEPES warmed to 37<sup>0</sup> C (except the wells containing PNP+A23187) and 200 microliters of plasma sample. The PNP+A23187 well sample consisted of 200 microliters of PNP, 10 microliters of the A23187 calcium ionophore, and 790 microliters of HEPES warmed to 37<sup>0</sup> C.

The macrophages immersed in their plasma solutions were then incubated at 5% CO<sub>2</sub> and 37<sup>0</sup>C for 2 hours. The well solutions were sampled at 30, 60, 90 and 120 minutes; 220 microliter samples were taken per well per sampling. The samples were stored in a biofreezer at -80<sup>0</sup> C until a radioimmunoassay (RIA) was performed. The RIA was performed using a standard commercially available RIA (Amersham) and a Beckman LS 3801 gamma-scintillation counter.

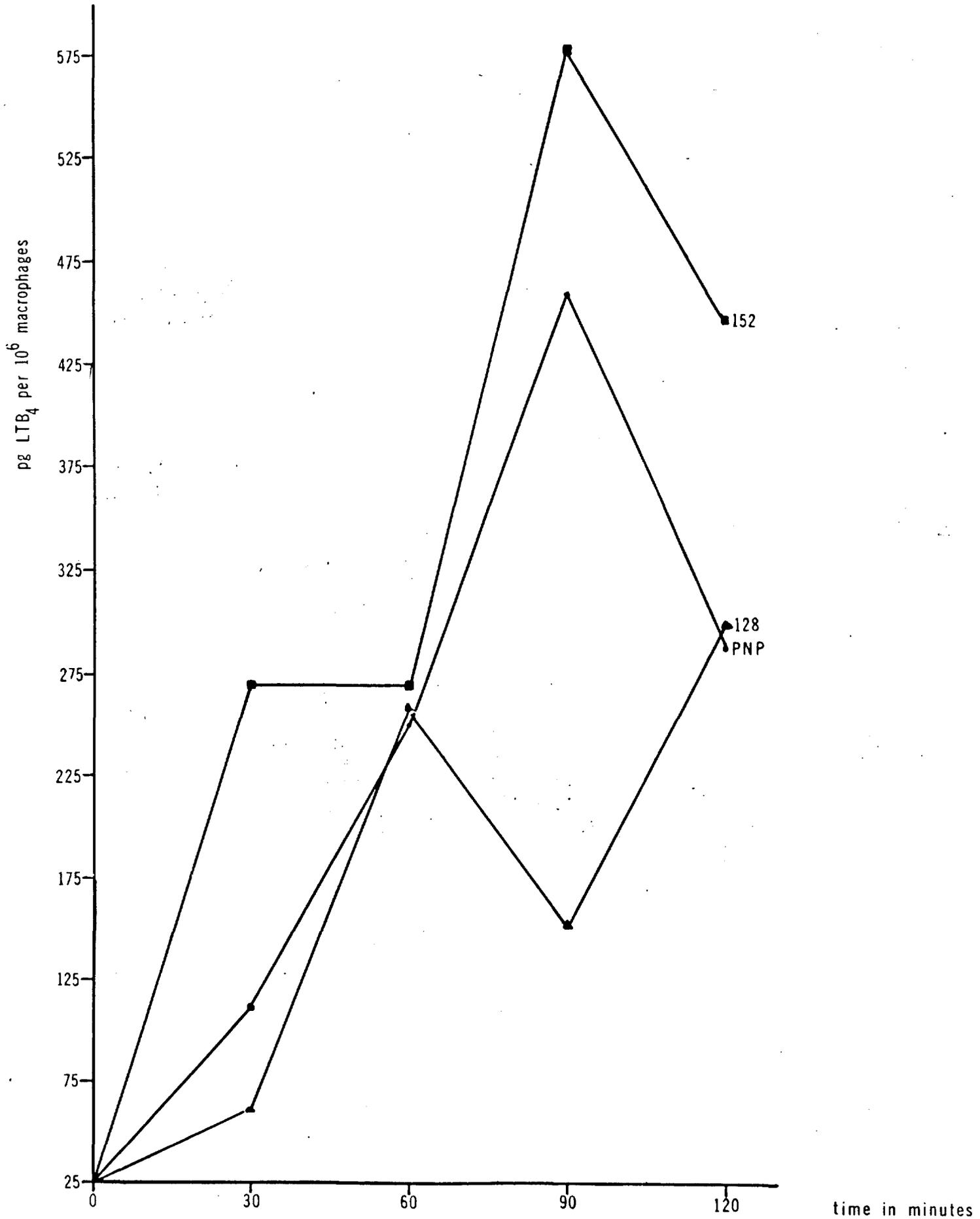
An RIA of 1:5 dilutions of each of the available IPP's (200 microliters IPP + 800 microliters HEPES) was performed to determine the background levels of LTB<sub>4</sub> in the IPP prior to their exposure to the macrophages.

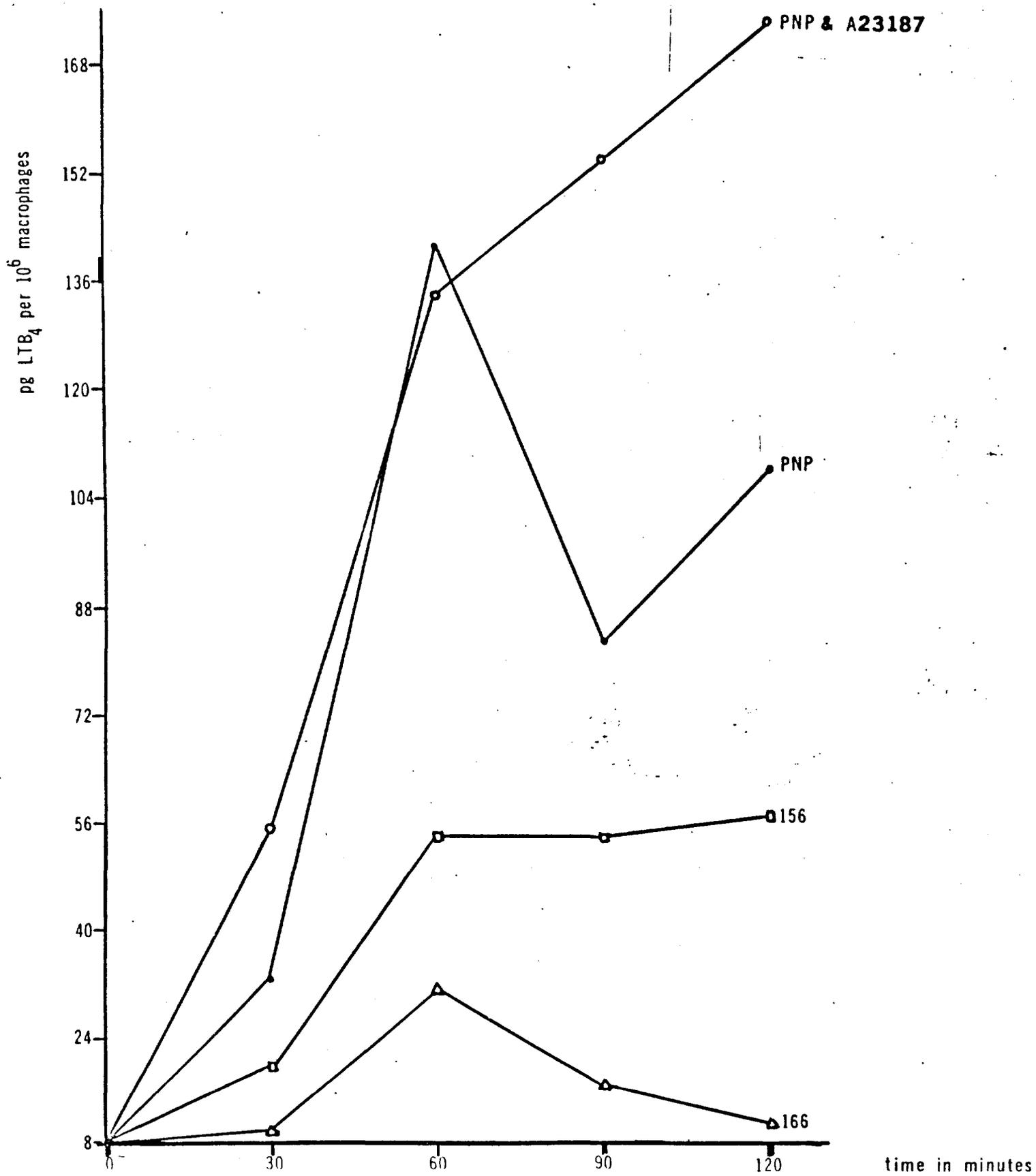
## **Results and Data:**

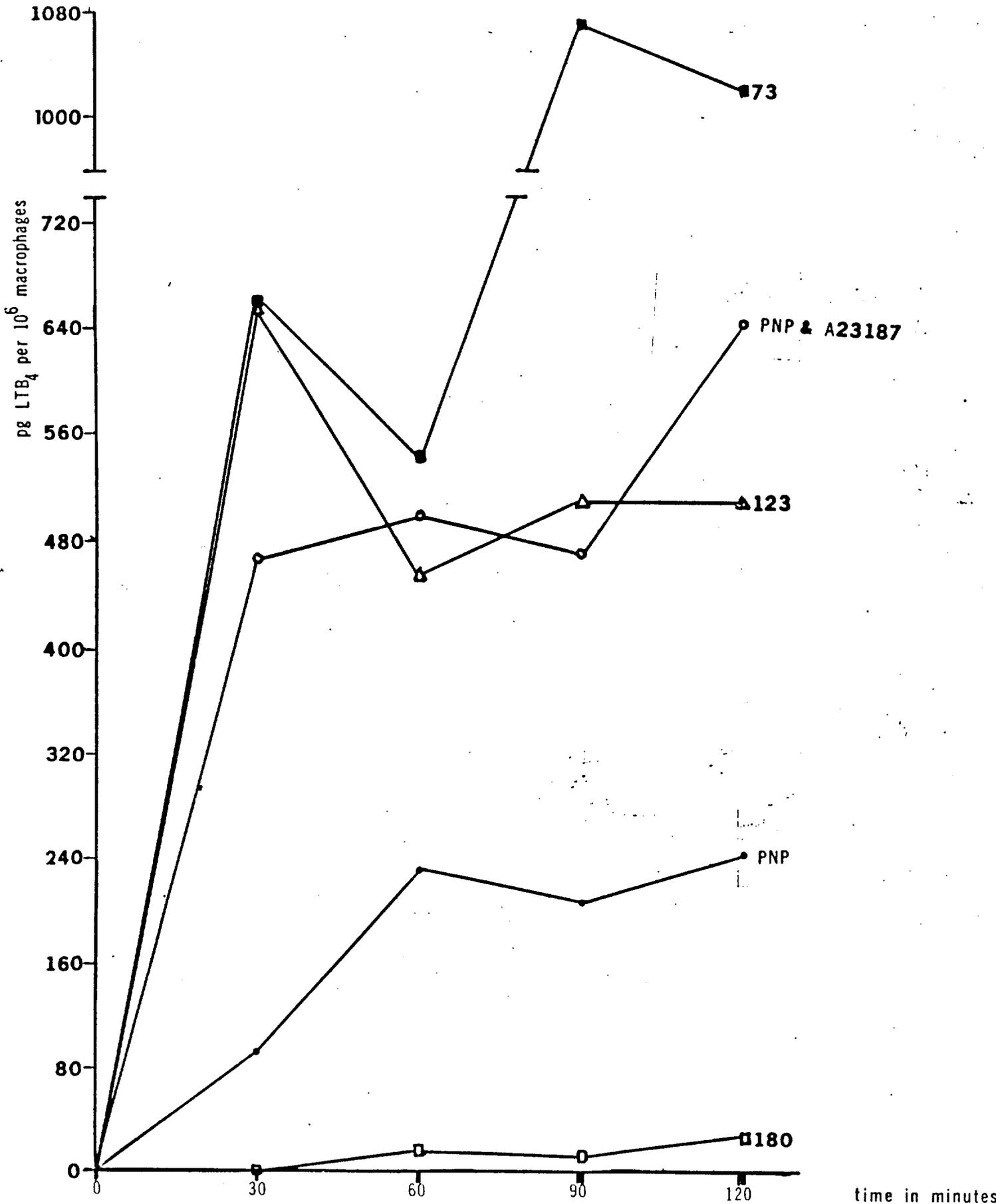
The peritoneal macrophages taken from adult BALB-C mice were allowed to adhere to the base of tissue-culture wells. The macrophages were then incubated while immersed in their respective plasma sample solutions. Plasma solution samples (120 microliters) were removed at 30, 60, 90 and 120 minutes. Each sample was then assayed via RIA to determine the total amount of LTB<sub>4</sub>. The background levels of LTB<sub>4</sub> obtained via RIA were then subtracted from the raw RIA data obtained previously. A background level for PNP was determined by averaging all of the background levels equal to less than 9 picograms (pg). The pg of LTB<sub>4</sub> per 10<sup>6</sup> macrophages were then calculated from the RIA data difference.

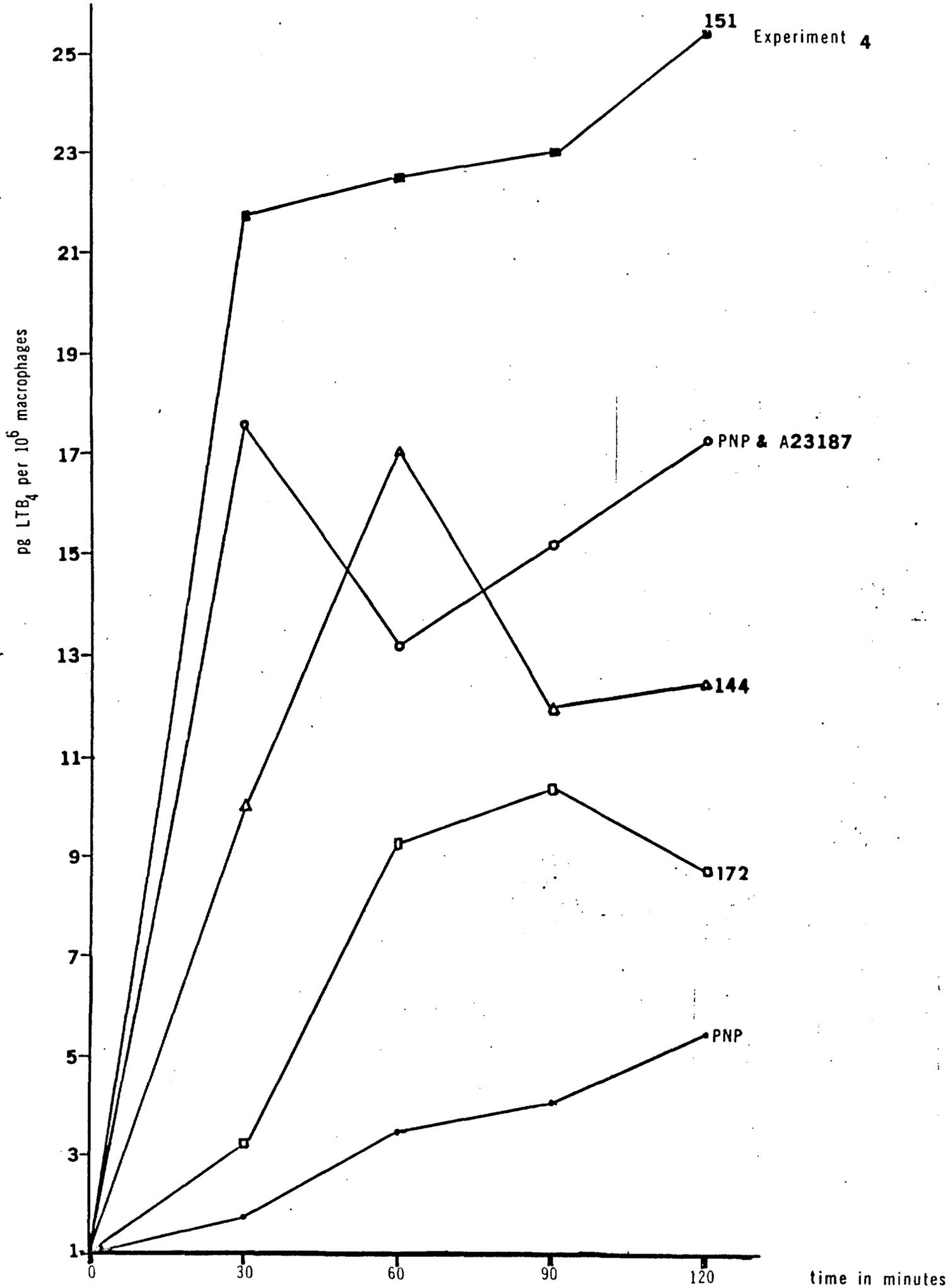
The results of each of the experiments (1 through 7) are shown in the following graphs. The results expressed in pg of LTB<sub>4</sub> per 10<sup>6</sup> macrophages vs. time in minutes.

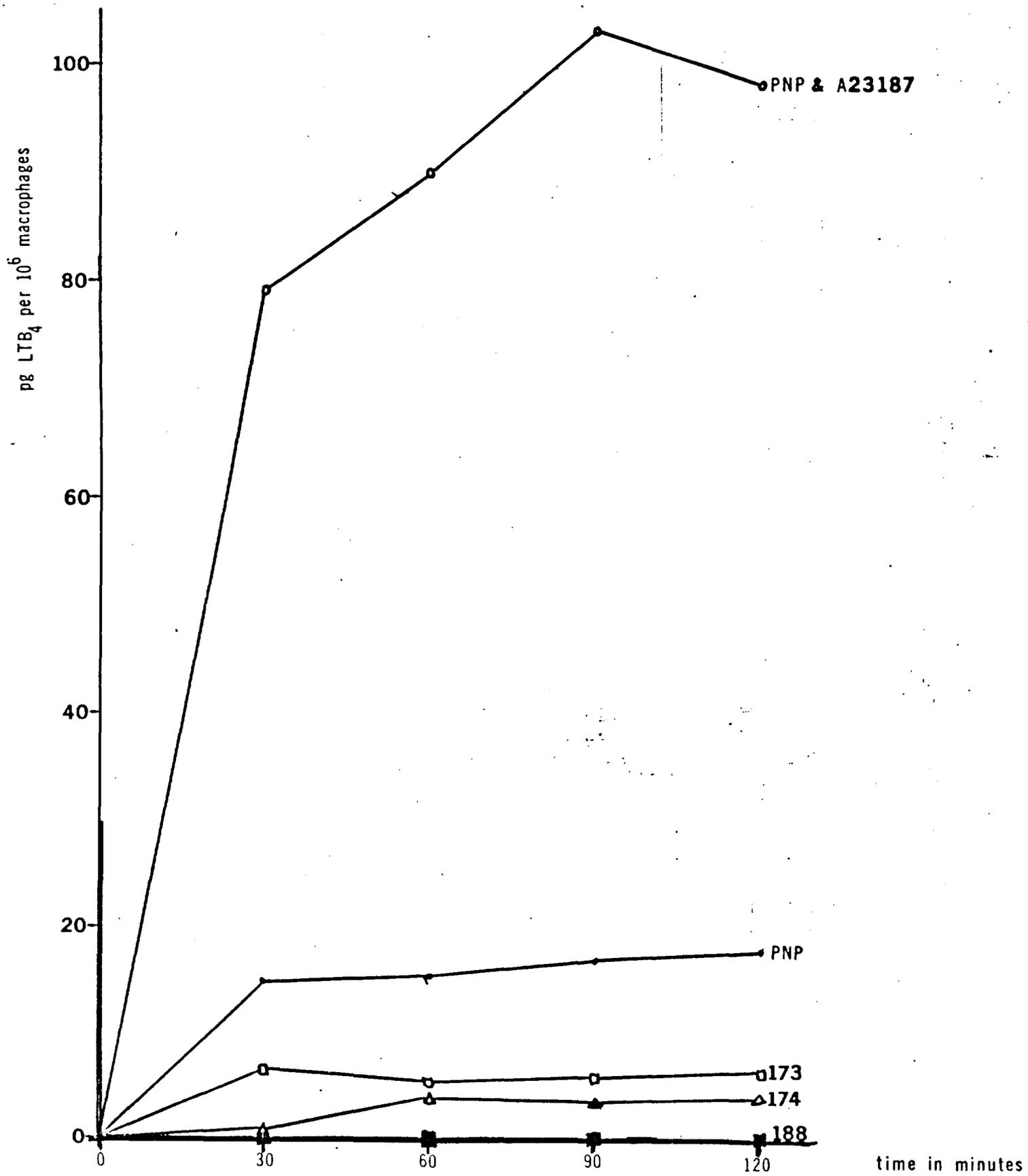
Experiment 1

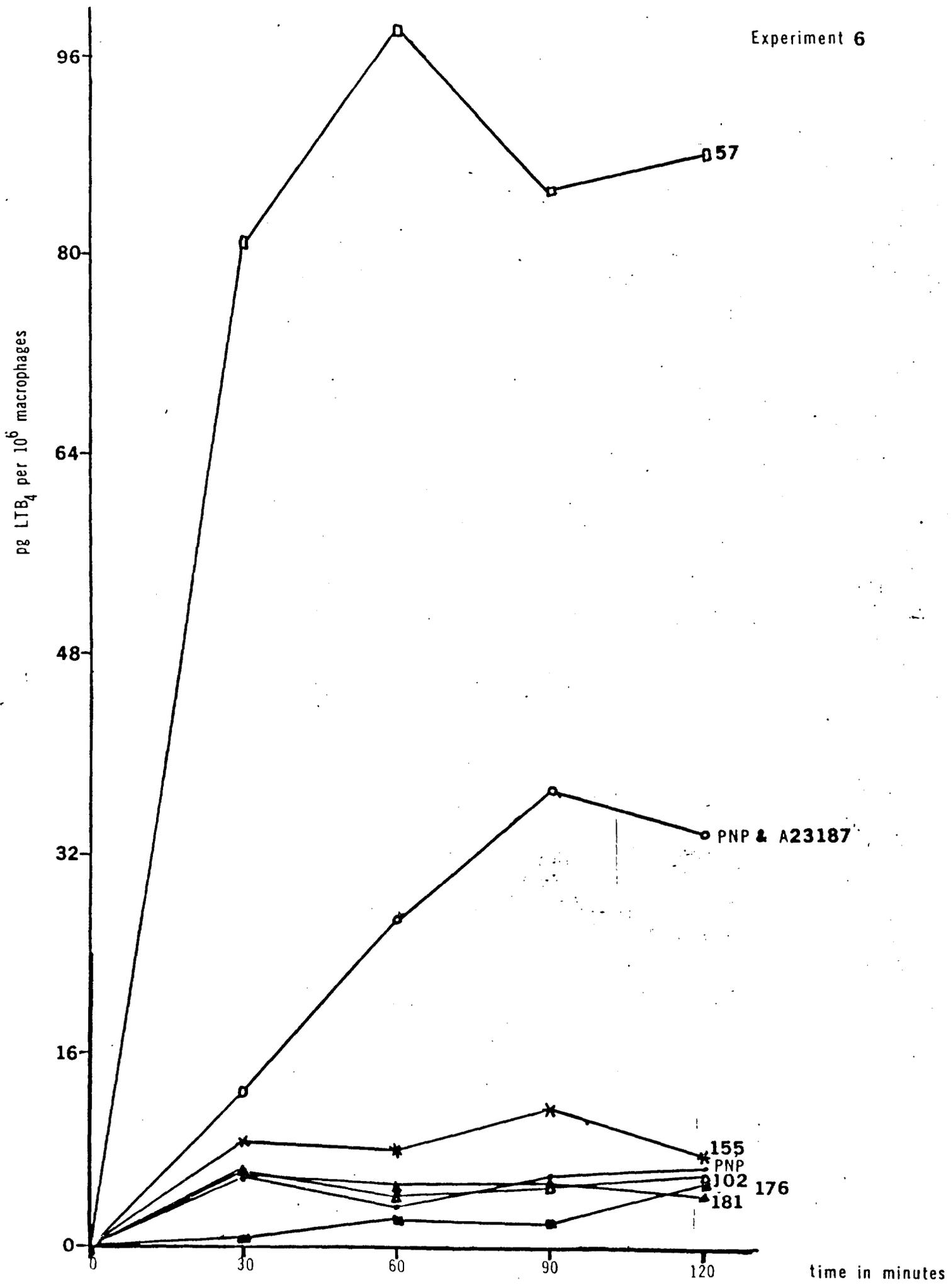


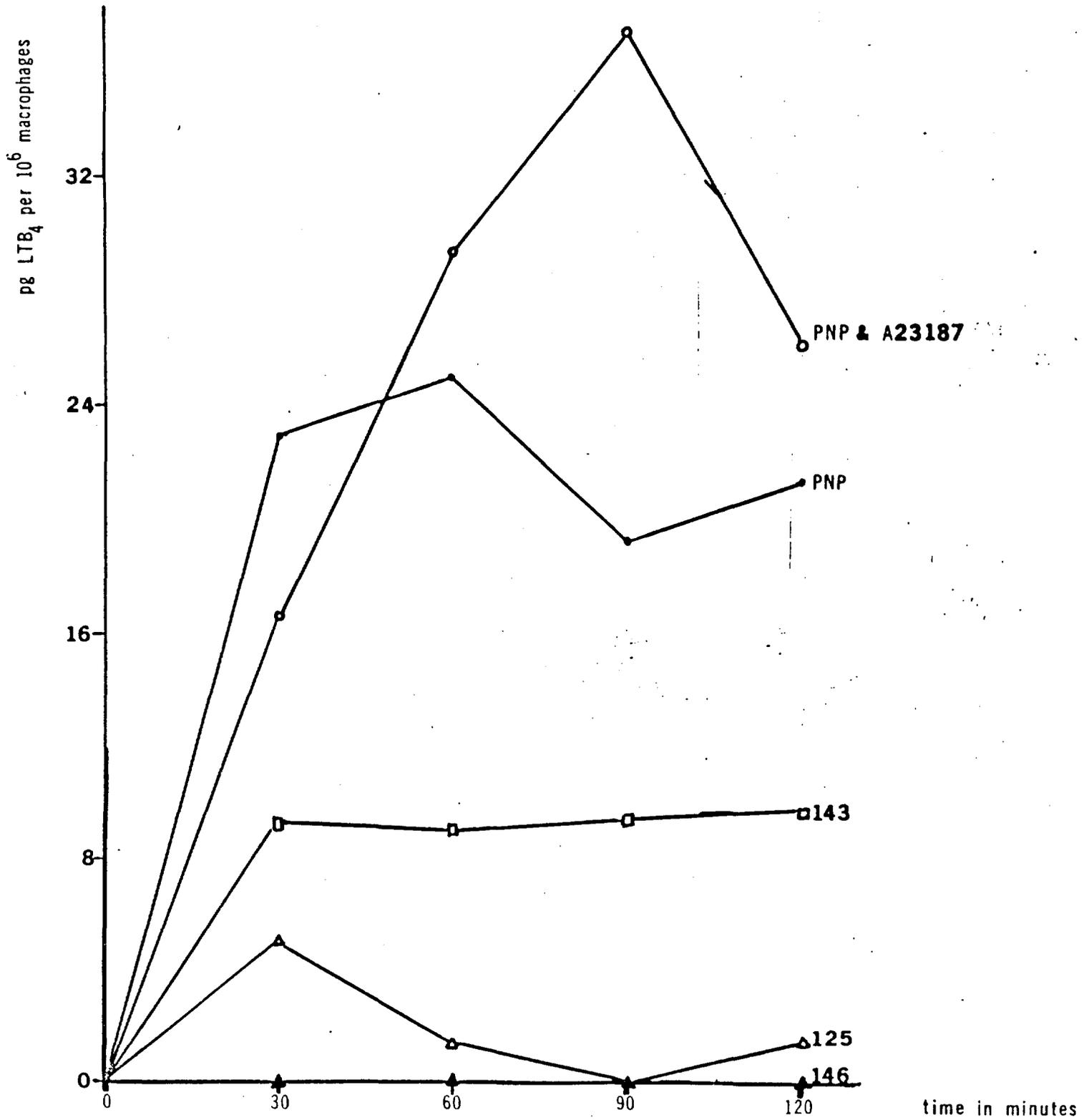












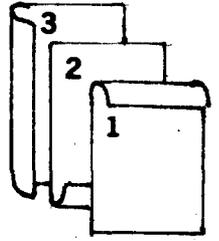
The degree of stimulation of the macrophages was held as constant as possible. However, there were deviations between experiments relative to stimulation. For this reason the pg of LTB<sub>4</sub> per 10<sup>6</sup> macrophages were expressed as a percentage of the pg of LTB<sub>4</sub> in the 120 minute PNP control sample. The results are shown on the experiments combined graph and in Table 1. The results are expressed as a percentage of pg of LTB<sub>4</sub> in the PNP control sample at 120 minutes vs. time in minutes. Overlay 1 displays the percentage values of all the male subjects, overlay 2 displays the percentage values of all the female subjects, and overlay 3 displays the percentage values of those patients for which there was limited medical history.

Table 1:

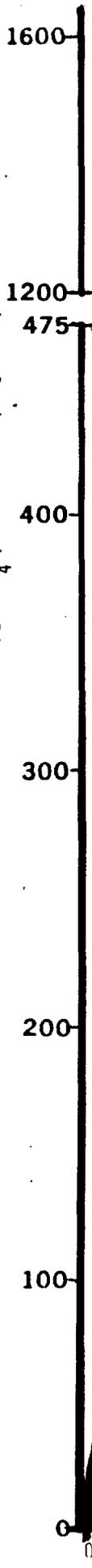
Sample percentages of 120 minute PNP-control sample.

<u>Sample</u>	<u>30 minutes</u>	<u>60 minutes</u>	<u>90 minutes</u>	<u>120 minutes</u>
PNP	59.32	91.27	96.14	100
PNP+A	213.37	271.42	324.02	323.04
57	1252.7	1527.9	1331.6	1339.4
73	269.81	222.75	437.83	428.55
102	94.70	65.11	82.40	92.06
123	268.48	187.38	209.24	209.24
125	23.97	6.53	2.66	6.53
128	21.38	89.97	53.20	102.61
143	43.28	42.02	43.75	45.06
144	184.03	309.44	217.06	226.68
146	0	0	0	0
151	395.28	407.80	417.97	461.52
152	128.32	128.32	199.99	155.45
155	137.54	127.26	182.87	121.81
156	18.84	50.36	49.89	53.04
166	9.23	28.89	16.09	10.83
172	58.98	168.24	188.93	158.62
173	36.65	30.95	34.64	38.10
174	5.75	22.35	19.22	21.23
176	12.62	38.16	35.05	90.03
180	0	7.1	5.68	11.05
181	95.48	78.66	91.90	69.31
188	0	0	0	0

Experiments  
combined



pg LTB<sub>4</sub> per 10<sup>6</sup> macrophages expressed as a % of 120' control PNP





Legend:

CL= anticardiolipin antibody  
PS= antiphosphatidyl antibody  
PA= antiphosphatidic acid antibody  
PI= antiphosphatidyl inositol antibody

The number of positives (+) indicates the relative amount of antiphospholipid antibody (APA) detected. Zero (0) represents no APA detected by ELISA.

The  $LTB_4$  data, the APA data, and the patients' medical histories were examined to determine any logical pattern which correlated abnormal levels of  $LTB_4$  to the presence of APA.  $LTB_4$  levels were considered abnormal if  $LTB_4$  secretion had been stimulated or inhibited. Inhibition was considered to be less than 50% of 120 minute PNP control percentage value. Alternately, stimulation was considered to be greater than 200% of the 120 minute PNP control percentage value.

Of the 18 patients' plasmas tested, 8 (3 female and 5 male) have drug-induced LA. Of the 8 drug-induced LA cases, 5 have abnormal  $LTB_4$  secretion. Furthermore, of the 8 drug-induced cases, a total of 5 have detectable levels of APA; 4 of those 5 also have abnormal  $LTB_4$  secretion. These results do not justify any reasonable correlation between abnormal  $LTB_4$  secretion and the presence of APA.

However, two of the 13 patients' plasmas tested do show reasonable correlation. The 2 female patients of child-bearing age (123 and 144) have stimulated  $LTB_4$  secretion, detectable APA levels, medical records of spontaneous abortion, negative rapid plasma reagin (RPR), and comparable APTT values. The similar attributes expressed by 123 and 144 imply a possible correlation between spontaneous abortions, the presence of LA, abnormal  $LTB_4$  secretion, and the presence of APA.

Overall, the plasma effects on  $LTB_4$  secretion do not appear to be the factor which causes thromboembolic disorders in patients with LA. There

does appear to be some evidence linking the level of  $\text{LTB}_4$  secretion and spontaneous abortions in young women. A study examining the plasma effects on  $\text{LTB}_4$  secretion and the presence of APA in a population of women of child-bearing age would be the next logical step.

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