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EFFECTS OF PROCARBAZINE HYDROCHLORIDE ON THE GUINEA PIG THYMUS GLAND AND IMMUNOSUPPRESSION TO DINITROCHLOROBENZENE SENSITIZATION

A DISSERTATION

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EFFECTS OF PROCARBAZINE HYDROCHLORIDE ON THE GUINEA PIG THYMUS GLAND AND IMMUNOSUPPRESSION TO DINITROCHLOROBENZENE SENSITIZATION

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EFFECTS OF PROCARBAZINE HYDROCHLORIDE ON THE GUINEA PIG THYMUS GLAND AND IMMUNOSUPPRESSION TO DINITROCHLOROBENZENE SENSITIZATION

CHAPTER I

INTRODUCTION

Procarbazine hydrochloride (PH), a methylhydrazine derivative, was originally introduced as an anti-neoplastic agent (6) and is considered one of the more active members of the group of synthetic cytotoxic compounds derived from methylhydrazine (12). The trade names for procarbazine hydrochloride have been Natulan, Matulane and in earlier publications it was referred to as methylhydrazine derivative, Ro-4-6467 or Ibenzmenthizine (77).

The mechanism of action of procarbazine is uncertain. *In vitro* studies (12) have demonstrated its ability to inhibit the synthesis of protein, RNA and DNA. *In vitro* analysis has demonstrated that cytotoxic derivatives of methylhydrazine can degrade DNA through a mechanism involving the auto-oxidation of the methylhydrazine to hydrogen peroxide (81). Berneis, *et al.*, (4) have proposed that procarbazine *in vivo* may break down to form formaldehyde, azomethine, N-hydroxymethyl derivatives and in agreement with *in vitro* studies the release of hydrogen peroxide. The *in vivo* growth inhibiting effects of procarbazine may be caused by both oxidation and alkylation of cellular constituents (4). Kreis and Yen (51)

have shown *in vivo* N-demethylation of procarbazine. Kreis (50) has also demonstrated a selective effect of the compound on the methylation of transfer RNA and has suggested that this may contribute to its carcinostatic activity.

Procarbazine has been demonstrated to be an effective anti-tumor agent in animals (6, 70) and in man. In man it has received emphasis when used alone (5, 7, 9, 12, 19, 20, 25, 26, 27, 39, 43, 61, 62, 80, 84, 87) or in combination with other (10, 11, 18, 19, 36, 56, 66, 78) chemotherapeutic agents for the treatment of Hodgkin's disease. The effectiveness of procarbazine in combination with radiotherapy for the treatment of Hodgkin's disease (2, 64, 75) and of solid tumors (52) has been demonstrated.

As with other anti-neoplastic drugs procarbazine is also known to induce severe adverse reactions. Kelly, *et al.*, (48) and others (22, 69) have demonstrated the ability of procarbazine to induce neoplasms in animal models. Other adverse reactions which have been attributed to procarbazine hydrochloride treatment have included toxic effects on the hematopoietic tissue (30), the cardiovascular (42), and the central nervous system (42) and its ability to induce allergic hypersensitivities (21, 47, 55).

Procarbazine hydrochloride is similar to other anti-tumor agents in that it has been demonstrated to be an effective immunosuppressive agent. The immunosuppressive characteristics of procarbazine hydrochloride have been demonstrated: a) to suppress the humoral immune response (77, 85); b) to suppress adjuvant induced autoimmune disease (77); c) to prolong acceptance of human renal transplants (73); d) to prolong survi-

val of xenografts (16, 44); e) to increase graft survival when there is pretreatment of graft donors (37, 38, 88, 89); f) to enhance immunosuppression when given in combination with anti-lymphocyte serum (31); g) to abrogate the graft-versus-host reaction (35).

Stewart and Bell (85), Floersheim, (29) and Possanza and Stewart (74), have demonstrated optimal immunosuppressive effects for PH when given for 21 days prior to exposure to the antigen. Quagliata, et al., (77) and Floersheim, et al., (32) have demonstrated the lymphotoxic effects of methylhydrazine derivatives on cortical thymic lymphocytes. In rats (57, 77) and mice (29, 86), PH has been reported to suppress or completely abolish thymus-dependent but not thymus-independent immunity. Although the immunosuppressive and tolerance levels of PH have been reported for rats, mice, and rabbits (85), comparable information has not been reported for guinea pigs.

Taking into consideration the importance of pretreatment, thymus destruction and the effectiveness of PH on thymus-dependent immunity, the present study was designed with the following objectives: a) to determine an effective immunosuppressive dose level of PH for guinea pigs; b) to determine by light and electron microscopy the sequential morphological alterations of the thymus during PH treatment; c) to demonstrate by dinitrochlorobenzene skin sensitization the degree of cell-mediated immunosuppression induced by chronic PH treatment; d) to correlate the recovery from immunological suppression with the morphological changes in the thymus.

CHAPTER II

MATERIALS, METHODS AND EXPERIMENTAL DESIGN

Materials

Animals

Hartley strain female guinea pigs obtained from Camm Research Institute, Inc., (414 Black Oak Ridge Road, Wayne, New Jersey) were utilized in the following studies. Upon arrival, the young adult animals weighing 400-450 grams were placed on a routine diet and housed in semi-isolation for at least one week before assignment to study groups. Once assigned, all animals were weighed daily and examined for overt manifestations of disease.

Prophylactic antibiotics were never administered to immunologically suppressed or to control animals.

Immunosuppressant

Procarbazine hydrochloride. The commercial name for the immunosuppressant procarbazine hydrochloride (PH) is Matulane (Hoffman-LaRoche, Inc., Nutley, New Jersey).

Skin Sensitizer and Skin Test Antigen

1-Chloro-2, 4-Dinitrobenzene. The skin sensitizer and skin test

antigen 1-chloro-2, 4-dinitrobenzene (DNCB) was obtained from Eastman Kodak Co., (Rochester, New York).

Methods

Preparation and Application of Procarbazine Hydrochloride

Procarbazine hydrochloride (PH). To yield the dosage levels required by a particular experimental protocol PH was quantitatively measured for each experimental animal and solubilized in 0.5 ml of sterile distilled water. This volume of the freshly prepared aqueous PH was injected subcutaneously into the dorsal scapular region of each test animal. The total number of injections was determined by the experimental design.

Evaluation of the Immune Response

Skin homografts. An area on the back of a donor guinea pig was shaved and the animal sacrificed by cervical dislocation. A full-thickness graft was removed and placed in a sterile petri dish containing Eagle's Minimum Essential Medium (Auto-Pow; Flow Laboratories) with 100 units of penicillin and 100 micrograms of streptomycin per ml (Gibco). The recipient was anesthesized with pentobarbitol (4 mg/100 gm body weight), the back shaved, and a 5/8 inch square skin graft bed exposed. A graft of comparable size was taken from the donor specimen, placed in the graft bed and secured with 5-0 suture.

Immunological skin testing. Both lumbar quadrants of all test animals were shaved 24 hours before the initial application of antigen. The skin sensitizing antigen consisted of a 2.0% solution of 1-chloro-2, 4-dinitrobenzene (DNCB) in absolute ethanol. Six drops of the sensitiz-

ing antigen, delivered through a 26 gauge needle, was topically applied to a single site within the shaved area. All sensitized animals received 7 daily applications of DNCB.

Skin testing was performed in the same general anatomical region however test sites were not superimposed on the site of DNCB sensitization. Twenty-four hours prior to skin testing, the area was reshaved and four individual test sites labeled. Test sites 1, 2, and 3 were for skin test evaluations at 24, 48, and 72 hours respectively. The skin test itself was performed by placing 1 drop of 0.5% DNCB dissolved in absolute ethanol on test sites 1, 2, and 3. Test site 4 was used as a control for the solvent and received 1 drop of pure absolute ethanol only.

Visual evaluation of skin test sites was performed at 24, 48, and 72 hours after application of the skin test antigen. The diameter of skin test sites was measured and graded for degree of induration. A grading system was devised to convert the degree of induration and mononuclear cell infiltration to a numerical value. A conversion scale is provided in Table 5.

Histological evaluation was performed on biopsy specimens taken from skin test sites 1, 2, and 3. Biopsies of test sites 1, 2, and 3 were taken at 24, 48, and 72 hours respectively and the specimens preserved for light microscopic examination.

Microscopic Examination

Thymus collection and preservation. Animals were sacrificed by cervical dislocation and both lobes of the thymus collected for light and electron microscopic examination. Each thymic lobe was divided into

equal parts by a parasagittal cut and one half of each lobe was processed for light microscopy. The remaining half of each lobe was prepared for electron microscopy.

Light microscopy. Tissues (thymus and skin) were fixed in 10% neutral buffered formalin and routinely processed in paraffin. Three micron sections were stained with hematoxylin and eosin.

Electron microscopy. The thymus half lobes (right and left) were fixed in 4% cacodylate buffered gluteraldehyde pH 7.4 for 24 hours at 4^{o} C. Following fixation the tissues were temporarily stored in sucrose buffer at 4^{o} C before being post-fixed in Palade's (72) osmium mixture for 1 hour. Tissues were then washed in veronal buffer, dehydrated in 70, 95, and 100% ethanol, cleared in propylene oxide and placed in a 1:1 mixture of propylene oxide and 6:4 Epon. Tissues were then infiltrated and embedded in pure 6:4 Epon. Gelatin capsules containing the Epon embedded tissues were polymerized for 24 hours at 60^{o} C.

Thick sections of Epon embedded material were used to verify the precise location of the EM specimen within the thymus gland, i.e., subcapsular, cortex, medulla, corticomedullary junction. Thick sections (2000 A) were heat dried on glass slides, stained with 1% crystal violet and examined light microscopically.

Thin sections producing a gold-silver interference color were cut on a Porter Blum MT 2 ultramicrotome. Sections were collected on #300 mesh copper grids, stained with uranyl acetate and lead citrate, and examined on a RCA EMU-3F electron microscope.

Experimental Design

The PH dose level tolerated by other animal species can not be correlated with their size or weight, therefore preliminary studies were necessary to determine both the tolerance (LD_{50}) and the optimum immunosuppressive levels for guinea pigs.

The following experiments utilizing animal Groups I and II were designed to obtain this information.

To facilitate the recording of complex treatment schedules and the arrangement of data in tabular form, the following numbering system was used throughout all subsequent studies. Numbers preceded by a minus sign (-) indicate the days of pretreatment with PH or water before evaluation of the immune response by skin grafting or skin sensitization. Numbers preceded by a plus sign (+) indicate the day or days of any procedure following pretreatment with PH or water, i.e., skin grafting, supplemental injections, DNCB sensitization, skin testing, or sacrifice.

Study I - Animal Group I

The 8 animals in Group I were divided into 4 equal subgroups, A, B, C, D. Experimental animals in subgroups A, B, and C received different dosage levels of PH solubilized in 0.5 ml of sterile distilled water; control animals in subgroup D received sterile water only. All animals were weighed daily and examined for gross toxic effects, e.g., physical signs of anemia (paleness of eyes and ears) and respiratory symptoms.

Experimental animals. Animals in subgroups A, B, and C received 15, 35, and 55 mg/Kg of PH respectively. After 21 daily injections (days -21 → -1), the degree of immunosuppression in surviving animals was eval-

uated by fullthickness skin homografts on day +1. Each experimental animal received three supplemental PH injections on days +2, +4, +6 before sacrifice on day +7.

Control animals. Subgroup D animals receiving 0.5 ml of sterile distilled water only followed the same time schedules of pretreatment, grafting, supplemental injections, and sacrifice as experimental animals.

The injection schedules, PH dosage levels and numbers of animals utilized in each group are summarized in Table 1.

Study II - Animal Group II

Information obtained from the previous study indicated that the optimum PH dosage level for guinea pigs was more than 15 mg/Kg but less than 35 mg/Kg. The results also indicated that the skin grafting procedure used for evaluating the immune response was unsatisfactory for the debilitated PH-treated animals.

In the following experiment, an intermediate dosage level of PH was selected, the number of supplemental injections was increased, and immunological skin testing was instituted for evaluating the degree of immunosuppression.

Experimental animals. PH treated animals in Group II-A received 25 mg/Kg in 0.5 ml of sterile water. After 21 daily injections, animals were sensitized on seven consecutive days (days +1 → +7) with topically applied DNCB. Experimental animals received six supplemental PH injections after the first day of exposure to DNCB. Supplemental injections were given every other day (days +2, +4, +6, +8, +10, +12) during and in the period immediately following DNCB sensitization. The effectiveness of this sequence of PH treatment in suppressing the immune response was

TABLE 1

STUDY I

PROTOCOL FOR DETERMINATION OF DOSE TOXICITY LEVELS TOLERATED BY GUINEA PIGS TREATED WITH PROCARBAZINE HYDROCHLORIDE

Animal Subgroups	Number of Animals	Dose of PH[a] (mg/Kg)[b]	Days Treated Prior to Skin Grafting	Day of Skin Grafting	Days of PH Treatment Following Skin Grafting	Day Sacrificed
I-A	2	15	-21 → -1	+1	+2 +4 +6	+7
I-B	2	35	-21 → -1	+1	+2 +4 +6	+7
I-C	2	55	-21 → -1	+1	+2 +4 +6	+7
I-D	2	None	-21 → -1	+1	+2 +4 +6	+7

[a] Procarbazine hydrochloride.

[b] Milligram per kilogram of whole body weight.

evaluated by skin testing 28 days after the first exposure to DNCB. Skin test sites were examined at 24 and 48 hours after application of the skin test antigen. Animals were sacrificed immediately after the 48 hour test reading and the thymus collected for histological examination.

Control animals. Group II-B animals receiving only distilled water were injected, immunized, skin tested, and sacrificed as were the experimental animals.

A detailed experimental protocol, including the animal numbers for Group II is summarized in Table 2.

Study III - Animal Groups III, IV, V

Results obtained from Study II allowed the maximum tolerated PH dosage of 25 mg/Kg, without undue toxicity, to be used in Studies III and IV. These animal groups were utilized only to evaluate the sequence of morphological changes occurring in the thymus during PH treatment. The letter "A" following a group number indicates PH treated animals receiving 25 mg/Kg in 0.5 ml sterile distilled water. The letter "B" following the group number indicated the corresponding control groups receiving 0.5 ml of sterile water only.

Experimental animals. Groups III-A, IV-A and V-A received daily injections of PH (25 mg/Kg) for 7, 14, or 21 days respectively. Animals in each group were sacrificed 24 hours following the last injection and the thymus preserved for light and electron microscopic examination.

Control animals. Groups III-B, IV-B, and V-B corresponding to the above experimental groups received daily injections of sterile water for 7, 14, or 21 days. Animals were sacrificed and the thymus collected as indicated above.

TABLE 2

STUDY II

PROTOCOL FOR EVALUATION OF TOXICITY AND IMMUNOSUPPRESSION IN GUINEA PIGS TREATED WITH 25 mg/Kg DOSES OF PROCARBAZINE HYDROCHLORIDE

Animal Groups	Number of Animals	Treatment with:	Days Treated Prior to DNCB[a] Sensitization	Skin Sensitization with DNCB on Days	Days Treated Following Initiation of DNCB Sensitization	Day of Skin Testing	Days of Skin Test Evaluations
II-A	8	PH[b]	-21 → -1	+1 → +7	+2 +4 +6 +8 +10 +12	+28	+29 +30 +31
II-B	4	H_2O	-21 → -1	+1 → +7	+2 +4 +6 +8 +10 +12	+28	+29 +31 +31

[a] 1-Chloro-2, 4-Dinitrobenzene.
[b] Procarbazine hydrochloride.

Table 3 contains the injection schedules for experimental and control animals in Groups III, IV, and V and includes the numbers of animals included in each group.

Study IV - Animal Groups VI, VII, VIII, IX

Study IV was designed to determine the duration of immunosupression and to correlate the immune recovery with sequential changes in thymic morphology. The duration and recovery rates were evaluated by skin testing DNCB sensitized animals at 0, 7, 14, and 21 days following the last exposure to the sensitizing antigen. All animals were sacrificed after the 72 hour skin test reading and the thymus preserved for light and electron microscopic examination.

Experimental animals. Groups VI-A, VII-A, VIII-A and IX-A received daily injections of PH (25 mg/Kg) for 21 days. Sensitization was begun 24 hours later when the animals received the first of seven daily applications of DNCB (days +1 → +7). During the seven days of sensitization, the experimental animals in all four groups received three additional PH injections on days +2, +4, +6. Experimental animals in Group VI-A were skin tested on the last day of sensitization to obtain a zero time interval. The remaining experimental groups received three additional PH injections on days +8, +10, +12 for a total of six supplemental injections. Animals in Groups VII-A, VIII-A, and IX-A were skin tested on days +14, +21, and +28 respectively to obtain the required time intervals of 7, 14, and 21 days.

Control animals. Groups VI-B, VII-B, VIII-B, and IX-B received water injections and followed the time schedules described for the corresponding experimental groups. Sensitization, skin test time intervals,

TABLE 3

STUDY III

SCHEDULE FOR SEQUENTIAL MICROSCOPIC EXAMINATION OF THYMUS TISSUE OF GUINEA PIGS TREATED WITH 25 mg/Kg DOSES OF PROCARBAZINE HYDROCHLORIDE

Animal Groups	Number of Animals	Treatment	Days Treated Prior to DNCB[a] Sensitization	Day Sacrificed
III-A	5	PH[b]	-7 → -1	+1
III-B	3	H_2O	-7 → -1	+1
IV-A	5	PH	-14 → -1	+1
IV-B	3	H_2O	-14 → -1	+1
V-A	5	PH	-21 → -1	+1
V-B	3	H_2O	-21 → -1	+1

[a] 1-Chloro-2, 4-Dinitrobenzene.
[b] Procarbazine hydrochloride.

evaluation and sacrifice procedure were identical to those of experimental animals.

A complete experimental protocol including animal numbers for each group is summarized in Table 4.

TABLE 4

STUDY IV

SCHEDULE FOR SEQUENTIAL EVALUATION OF IMMUNOSUPPRESSION

AND MICROSCOPIC EXAMINATION OF THYMUS TISSUE OF

GUINEA PIGS TREATED WITH 25 mg/Kg DOSES OF

PROCARBAZINE HYDROCHLORIDE

Animal Groups	Number of Animals	Treatment	Days Treated Prior to DNCB[a] Treatment	Days of DNCB Treatment	Days Treated Following Initiation of DNCB Exposure	Day Interval between Last Exposure to DNCB and Skin Testing	Day of Skin Testing	Days of Skin Test Evaluation
VI-A	5	PH[b]	-21 → -1	+1 → +7	+2 +4 +6	0	+7	+8 +9 +10
VI-B	3	H_2O	-21 → -1	+1 → +7	+2 +4 +6	0	+7	+8 +9 +10
VII-A	5	PH	-21 → -1	+1 → +7	+2 +4 +6 +8 +10 +12	7	+14	+15 +16 +17
VII-B	3	H_2O	-21 → -1	+1 → +7	+2 +4 +6 +8 +10 +12	7	+14	+15 +16 +17

TABLE 4--Continued

Animal Groups	Number of Animals	Treatment	Days Treated Prior to DNCB[a] Treatment	Days of DNCB Treatment	Days Treated Following Initiation of DNCB Exposure	Day Interval between Last Exposure to DNCB and Skin Testing	Day of Skin Testing	Days of Skin Test Evaluation
VIII-A	5	PH	-21 → -1	+1 → +7	+2 +4 +6 +8 +10 +12	14	+28	+22 +23 +24
VIII-B	3	H_2O	-21 → -1	+1 → +7	+2 +4 +6 +8 +10 +12	14	+28	+22 +23 +24
IX-A	10	PH	-21 → -1	+1 → +7	+2 +4 +6 +8 +10 +12	21	+28	+29 +30 +31
IX-B	5	H_2O	-21 → -1	+1 → +7	+2 +4 +6 +8 +10 +12	21	+28	+29 +30 +31

[a] 1-Chloro-2, 4-Dinitrobenzene.
[b] Procarbazine hydrochloride.

TABLE 5

NUMERICAL GRADING SYSTEM FOR DEGREE OF INDURATION AND MONONUCLEAR CELL INFILTRATION OF SKIN TEST SITES

Degree of Induration	Assigned Numerical Value	Degree of Mononuclear Cell Infiltration
None Observed	0.00	None Observed
"Light"-Light	1.25	"Light"-Light
"Medium"-Light	1.50	"Medium"-Light
"Strong"-Light	1.75	"Strong"-Light
"Light"-Medium	2.25	"Light"-Medium
"Medium"-Medium	2.50	"Medium"-Medium
"Strong"-Medium	2.75	"Strong"-Medium
"Medium"-Strong	3.50	"Medium"-Strong
"Strong"-Strong	3.75	"Strong"-Strong

CHAPTER III

RESULTS

Experimental and control animals were evaluated by physical, immunological and histological means. All animals were weighed and examined daily for signs of illness and the PH treated animals were observed closely for signs of drug toxicity, i.e., suppressed weight gain, indirect evidence of anemia (paleness and extramedullary hematopoiesis), respiratory distress, lethargy and death.

Toxic Effects of Procarbazine Hydrochloride Treatment

Most PH treated animals displayed some evidence of drug toxicity. The earliest physical manifestation of toxicity was weight change which became more severe as the dosage level was increased. Additional toxic effects, anemia and respiratory symptoms, appeared somewhat later and could be correlated with the dose level and with the duration of treatment. Early stages of anemia and respiratory distress appeared to be reversible and usually disappeared after completion of the PH injection sequence, but the later stages were frequently irreversible. High levels of PH administered for prolonged periods produced more devastating effects progressing to lethargy and terminating in death.

Weight Changes

Study I. Three PH dosage levels (15, 35, and 55 mg/Kg) were uti-

lized in an attempt to determine both the tolerance and acceptable immunosuppressive levels for guinea pigs. Animals receiving 15 mg/Kg exhibited no physical signs of toxicity except for a slight reduction in the rate of weight gain (growth). Experimental animals receiving 35 and 55 mg/Kg were overtly toxic and exhibited an actual weight loss rather than a reduction in the rate of weight gain. Animals receiving the highest dosage levels (55 mg/Kg) did not survive the anesthesization procedure required for full thickness skin homografts.

Study II. An intermediate PH dosage level of 25 mg/Kg was used in an attempt to reduce overt toxicity while maintaining an adequate level of immunosuppression. Experimental animals exhibited a marked decrease in the rate of weight gain when compared to control animals (Table 6). Although this dosage produced a marked reduction in the rate of weight gain, the treated animals exhibited minimal evidence of other toxic symptoms.

Studies III and IV. After the first week of PH treatment, all experimental animals receiving 25 mg/Kg exhibited a continuous weight gain, but the average increase was less than that exhibited by the corresponding control groups (Table 7). During the first week however, experimental animals in groups IV-A, V-A, and VII-A and the control animals in group V-B exhibited a transitory weight loss. Animals exhibiting a prolonged and severe weight loss almost invariably died prior to completion of the PH injection schedule.

Mortality

In studies III and IV, in which the experimental animals received 25 mg/Kg, some deaths occurred (15%) (Table 8). All deaths occurred in

TABLE 6

WEIGHT CHANGE OF ANIMALS IN STUDY II TREATED WITH

25 mg/Kg DOSES OF PROCARBAZINE HYDROCHLORIDE

Animal Group		Days of Experiment -1	-7	-14	-21	+7	+14	+21	+28
II-A	a	467	469	499	509	514	526	539	532
	b	0	+2	+32	+42	+47	+59	+72	+65
II-B	a	453	476	517	547	574	608	614	614
	b	0	+23	+64	+94	+121	+155	+161	+161

[a] Mean body weight on days indicated.
[b] Mean weight change from initial average weight on day -1.

TABLE 7

WEIGHT CHANGE OF ANIMALS IN STUDIES III AND IV TREATED WITH 25 mg/Kg DOSES OF PROCARBAZINE HYDROCHLORIDE

Animal Groups		Days of Experiment -1	-7	-14	-21	+7	+14	+21	+28
III-A	a	403	403						
	b	0	0						
III-B	a	415	432						
	b	0	+17						
IV-A	a	416	400	450					
	b	0	-16	+34					
IV-B	a	435	440	509					
	b	0	+5	+74					
V-A	a	406	389	415	430				
	b	0	-17	+9	+24				
V-B	a	416	414	465	490				
	b	0	-2	+49	+74				
VI-A	a	435	457	504	523	506			
	b	0	+22	+69	+88	+71			
VI-B	a	408	453	511	533	564			
	b	0	+45	+103	+125	+156			
VII-A	a	442	439	447	455	454	465		
	b	0	-3	+5	+13	+12	+23		
VII-B	a	425	431	476	503	545	569		
	b	0	+6	+51	+78	+120	+144		
VIII-A	a	425	428	461	475	478	469	496	
	b	0	+3	+36	+50	+53	+44	+71	
VIII-B	a	475	516	540	591	632	672	700	
	b	0	+41	+65	+116	+157	+197	+225	
IX-A	a	492	493	514	525	523	513	536	555
	b	0	+1	+22	+33	+31	+21	+44	+63
IX-B	a	483	519	548	598	629	667	701	723
	b	0	+36	+65	+115	+146	+184	+218	+240

[a] Mean body weight on days indicated.
[b] Mean weight change from initial average weight on day -1.

TABLE 8

MORTALITY OF ANIMALS IN STUDIES III AND IV

Animal Groups	Number of PH Injections	Duration of Study (days)	Number of Animals in Each Group	Number of Animals Dying	Number of Animals Living
III-A	7	7	5	0	5
III-B	0	7	3	0	3
IV-A	14	14	5	0	5
IV-B	0	14	3	0	3
V-A	21	21	5	1	4
V-B	0	21	3	0	3
VI-A	24	31	5	1	4
VI-B	0	31	3	0	3
VII-A	27	38	5	0	5
VII-B	0	38	3	0	3
VIII-A	27	45	5	1	4
VIII-B	0	45	3	0	3
IX-A	27	52	10	3	7
IX-B	0	52	5	0	5

animals receiving 21 or more PH injections. Of these deaths, two-thirds occurred in animals undergoing the longest duration of treatment (groups VIII-A and IX-A). No deaths occurred in the control animal groups.

Evaluation of Immunity

Homografts

Study I. The method, full thickness skin homografts, used to evaluate the effectiveness of PH immunosuppression was found to be unsatisfactory for the debilitated PH treated animals.

Skin Testing

Study II. In this study, skin testing of DNCB sensitized animals was instituted as the method for evaluating the effectiveness of PH as an immunosuppressant of cell-mediated immunity. Skin tests performed on experimental animals treated with 25 mg/Kg of PH indicated a marked immunosuppression (Table 2). When compared to control animals, the skin test diameters of PH treated animals were decreased by 20-30% at 24 hours and 40-60% at 48 hours (Table 9). The PH treated animals also exhibited a degree of skin test induration which was approximately 53% and 57% less than the controls at 24 and 48 hours respectively (Table 10).

Since experimental animals receiving 25 mg/Kg of PH exhibited marked immunosuppression while manifesting minimal degrees of drug toxicity, this dosage level was utilized in Studies III and IV to produce the suppressed state. Furthermore, since skin testing proved to be a satisfactory method for evaluating the degree of immune suppression, this method was retained for assessing the rate of immune recovery in Study IV.

Study IV. Skin test sites in animal groups VI, VII, VIII, and

TABLE 9

RESULTS OF SKIN TEST DIAMETERS IN ANIMALS IN STUDY II TREATED WITH 25 mg/Kg DOSES OF PROCARBAZINE HYDROCHLORIDE

Animal Group	Time Interval between Last Day of DNCB Sensitization and Time of Skin Testing	Percent Decrease between PH and Water Treated Animals at:[a]		Accumulative Percent Decrease between PH and Water Treated Animals at:
		24 hrs.	48 hrs.	(24 + 48 hrs.)
II	21	23	42	32

[a] Mean values of all animals in designated group: Mean of Group A divided by Mean of Group B.

TABLE 10

RESULTS OF SKIN TEST INDURATION IN ANIMALS OF STUDY II TREATED

25 mg/Kg DOSES OF PROCARBAZINE HYDROCHLORIDE

Animal Group	Time Interval between Last Day of DNCB Sensitization and Time of Skin Testing	Percent Decrease between PH and Water Treated Animals at:[a,b]		Accumulative Percent Decrease between PH and Water Treated Animals (24 + 48 hrs.)
		24 hrs.	48 hrs.	
II	21	53	57	55

[a] Mean values of all animals in designated group: Mean of Group A divided by Mean of Group B.

[b] Numerical values used to calculate the differences in degrees of induration were obtained from table 5 which converts the subjective grading to a numerical one.

IX were evaluated for diameter of erythema, for degree of induration, and for mononuclear cell infiltration.

Using the accumulative mean percentage values for the experimental and for the control animals, the greatest difference between skin test diameters occurred when the animals were skin tested at day 0 time interval post-sensitization (Table 11); the greatest difference in degree of induration occurred when the animals were skin tested at day 14 post-sensitization (Table 12) (Figures 1-6); the greatest difference between degree of mononuclear cell infiltration occurred when the animals were skin tested at day 7 post-sensitization (Table 13) (Figures 7-12).

Microscopic Examination of Thymus Tissue

Light Microscopy - Study III

Tissue for microscopic evaluation was obtained from animals groups III, IV, and V. These animals received PH or water for 7, 14, or 21 days prior to sacrifice.

Control animals. The thymic cortex of control animals receiving 7, 14, or 21 daily injections of water were thick and crowded with thymic lymphocytes (Figures 13, 15, 17, 23, 25, 27). The cellular population consisted of a mixture of small, medium, and large sized lymphocytes (Figures 23, 25, 27, 33, 35, 37) and contained an impressive number of large cortical lymphocytes (Figures 33, 35, 37).

The corticomedullary junction of control animals was distinct and sharply delineated (Figures 13, 15, 17).

Medullary regions of the control animals contained typical thymic corpuscles and were composed of a mixture of epithelial and lymphoid cells (Figures 43, 45, 47).

TABLE 11

RESULTS OF SKIN TEST DIAMETERS IN ANIMALS OF STUDY IV TREATED WITH 25 mg/Kg DOSES OF PROCARBAZINE HYDROCHLORIDE

Animal Group	Time Interval between Last Day of DNCB Sensitization and Time of Skin Testing	Percent Decrease between PH and Water Treated Animals at:[a]			Accumulative Percent Decrease between PH and Water Treated Animals at: (24 + 48 + 72 hrs.)
		24 hrs.	48 hrs.	72 hrs.	
VI	0	36	29	54	41
VII	7	+5*	21	15	10
VIII	14	30	25	22	26
IX	21	26	14	7	16

[a] Mean values of all animals in designated group: Mean of Group A divided by Mean of Group B.

* This value designates an increase rather than a decrease.

TABLE 12

RESULTS OF SKIN TEST INDURATION IN ANIMALS OF STUDY IV TREATED WITH 25 mg/Kg DOSES OF PROCARBAZINE HYDROCHLORIDE

Animal Group	Time Interval between Last Day of DNCB Sensitization and Time of Skin Testing	Percent Decrease between PH and Water Treated Animals at:[a,b]			Accumulative Percent Decrease between PH and Water Treated Animals at: (24 + 48 + 72 hrs.)
		24 hrs.	48 hrs.	72 hrs.	
VI	0	30	37	71	46
VII	7	38	10	27	25
VIII	14	48	53	61	54
IX	21	45	49	45	46

[a] Mean values of all animals in designated group: Mean of Group A divided by Mean of Group B.

[b] Numerical values used to calculate the differences in degrees of induration were obtained from Table 5 which converts the subjective grading to a numerical one.

TABLE 13

RESULTS OF SKIN TEST CELLULAR INFILTRATION IN ANIMALS OF STUDY IV

TREATED WITH 25 mg/Kg DOSES OF PROCARBAZINE HYDROCHLORIDE

Animal Groups	Time Interval between Last Day of DNCB Sensitization and Time of Skin Testing	Percent Decrease between PH and Water Treated Animals at:[a,b]			Accumulative Percent Decrease between PH and Water Treated Animals at: (24 + 48 + 72 hrs.)
		24 hrs.	48 hrs.	72 hrs.	
VII	7	67	45	27	46
VIII	14	+11*	11	22	7
IX	21	28	17	8	18

[a] Mean values of all animals in designated group: Mean of Group A divided by Mean of Group B.

[b] Numerical values used to calculate the differences in degrees of cellular infiltration were obtained from Table 5 which converts the subjective grading to a numerical one.

* This value designates an increase rather than a decrease.

Figure 1. Representation of induration graded as "None-Observed" (Numerical conversion value of 0.00). No skin test activity is demonstrable at test sites 1, 2, or 3. The control test site (ST 4) is covered by the animal label. The dark areas at ST 1, ST 2, and ST 3 are India ink used to mark test sites.

Figure 2. Representation of induration graded as "Medium"-Light (Numerical conversion value of 1.50). Skin test sites 2 and 3 demonstrate "medium"-light induration. The brown color at ST 4 is India ink. St 1 was biopsied at 24 hours and is covered with the animal label.

Figure 3. Representation of induration graded as "Strong"-Light (Numerical conversion value of 1.75). Test sites 1, 2, and 3 demonstrate "strong"-light induration which is best observed at ST 2. The control site (ST 4) is covered with the animal label.

Figure 4. Representation of induration graded as "Medium"-Medium (Numerical conversion value of 2.50). Test sites 1, 2, and 3 demonstrate "medium"-medium induration which is best observed at ST 2 and ST 3. The brown color at sites 2, 3 and 4 are India ink.

Figure 5. Representation of induration graded as "Strong"-Medium (Numerical conversion value of 2.75). Test sites 1, 2, and 3 demonstrate "strong"-medium induration. The dark color at the control site (ST 4) is India ink.

Figure 6. Representation of induration graded as "Medium"-Strong (Numerical conversion value of 3.50). Skin test sites 1, 2, and 3 demonstrate "medium"-strong induration which is best observed at ST 2. The dark areas adjacent to sites ST2 and ST 4 are India ink. The sensitizing site (SS) is also shown in this figure.

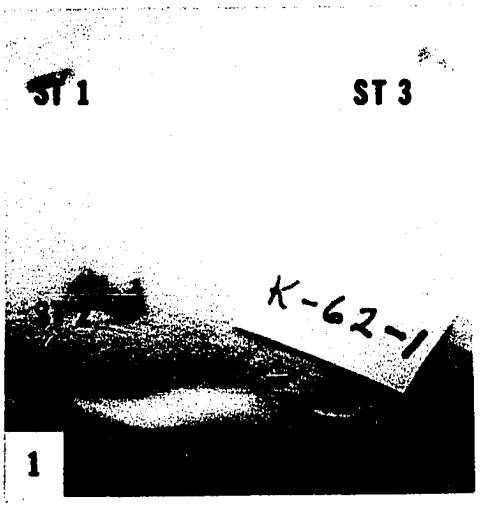
LEGEND

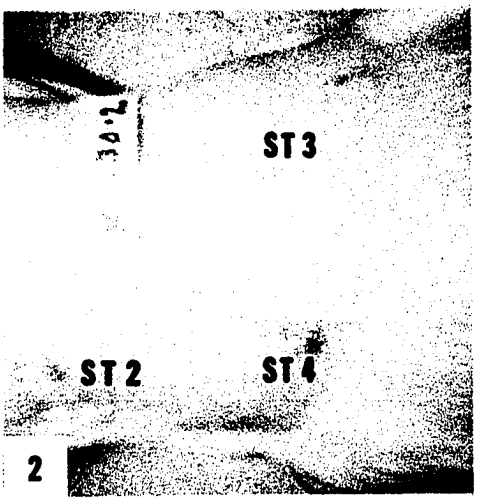
ST 1 Skin test site biopsied at 24 hours.

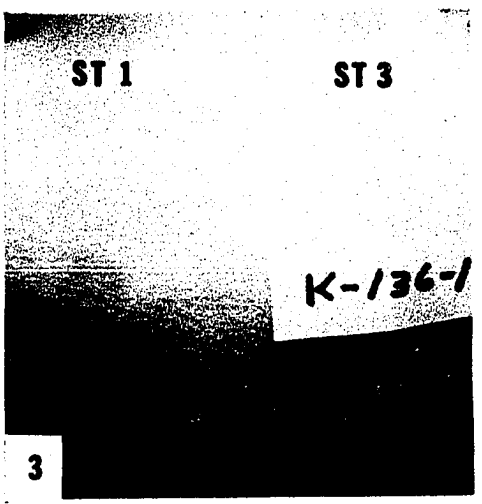
ST 2 Skin test site biopsied at 48 hours.

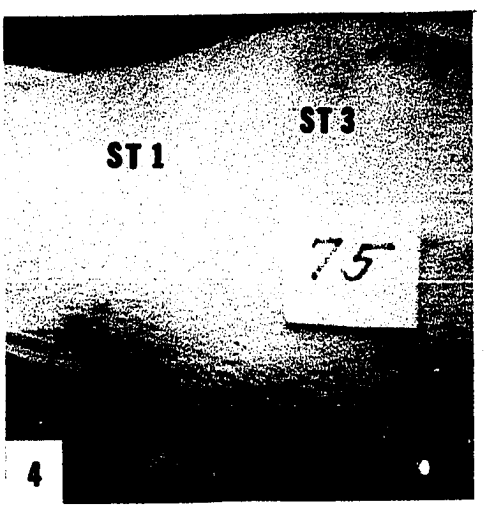
ST 3 Skin test site biopsied at 72 hours.

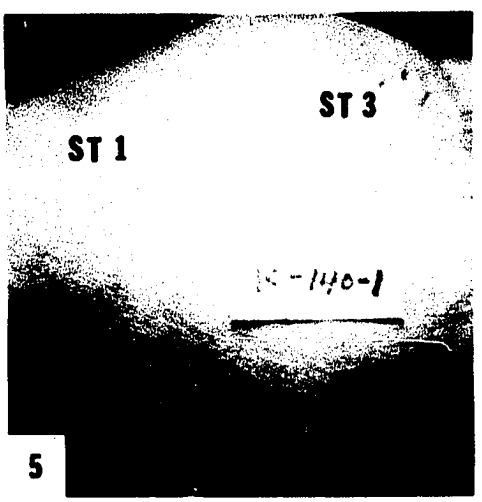
SS Sensitizing site.


ST 1
ST 3
K-62-1
1


ST 3
ST 2
ST 4
2


ST 1
ST 3
K-136-1
3


ST 3
ST 1
75
4


ST 3
ST 1
K-140-1
5

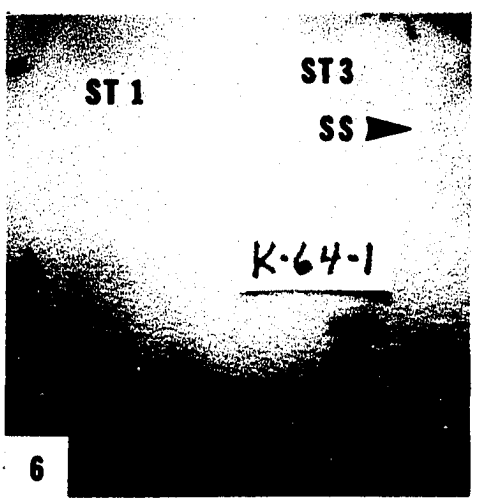

ST 1
ST 3
SS
K-64-1
6

Figure 7. Representation of mononuclear cell infiltration graded as "Medium"-Light (Numerical conversion value of 1.50). H&E 170X

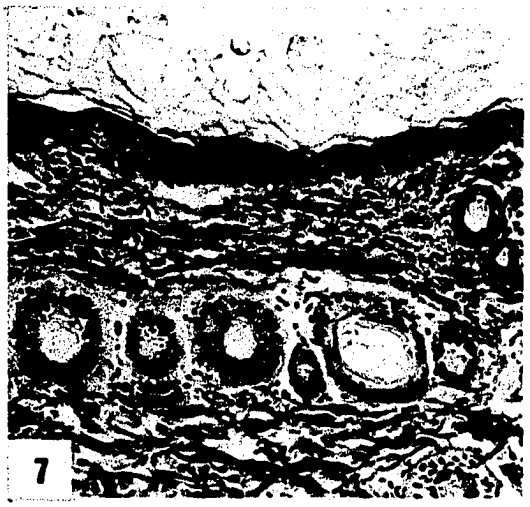
Figure 8. Representation of mononuclear cell infiltration graded as "Strong"-Light (Numerical conversion value of 1.75). H&E 170X.

Figure 9. Representation of mononuclear cell infiltration graded as "Medium"-Medium (Numerical conversion value of 2.50). H&E 170X.

Figure 10. Representation of mononuclear cell infiltration graded as "Strong"-Medium (Numerical conversion value of 2.75). H&E 170X.

Figure 11. Representation of mononuclear cell infiltration graded as "Medium"-Strong (Numerical conversion value of 3.50). H&E 170X.

Figure 12. Representation of mononuclear cell infiltration graded as "Strong"-Strong (Numerical conversion value of 3.75). H&E 170X.



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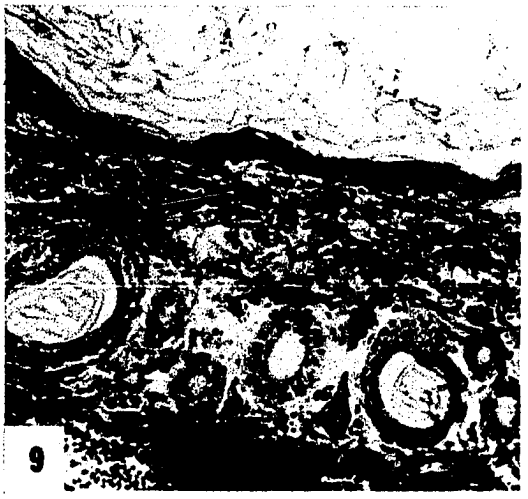

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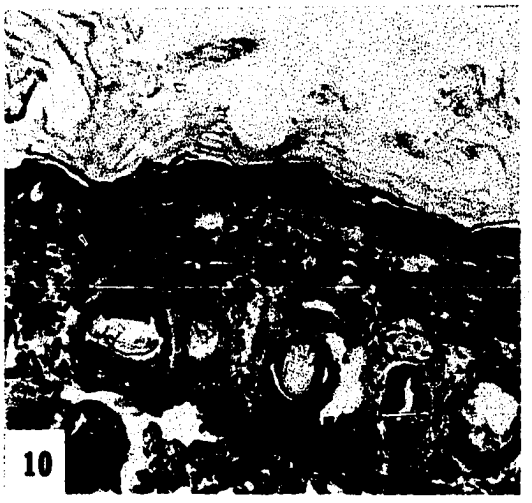

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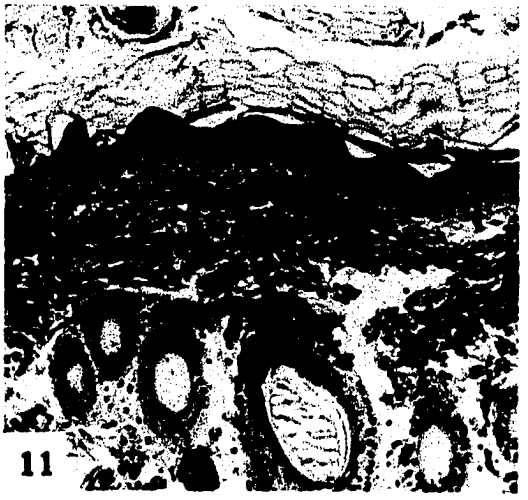

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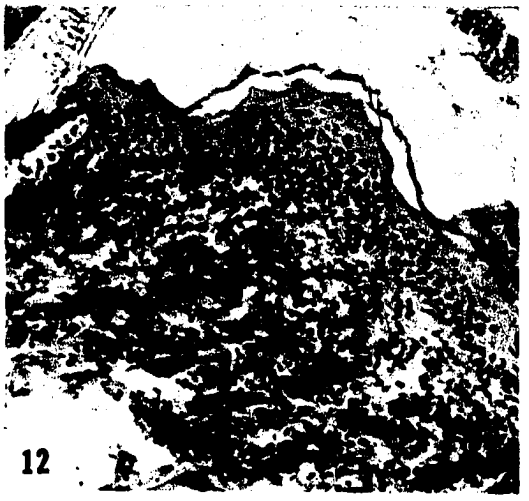

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Experimental animals. The thymic cortex of animals receiving 7, 14, or 21 daily injections of PH were depleted of thymic lymphocytes (Figures 14, 16, 18, 24, 26, 28, 34, 36, 38). As the duration and number of PH injections increased, the severity of lymphocyte depletion increased (Figures 14, 16, 18, 24, 26, 28). Depletion was particularly pronounced in animals receiving PH for 21 days (Figures 18, 28). Cortical depletion involved small, medium, and large sized lymphocytes (Figures 24, 26, 28, 34, 36, 38).

Corticomedullary junctions in PH treated animals were somewhat diffuse and difficult to define (Figures 14, 16, 18, 24, 26, 28).

The medullary regions of PH treated animals did not exhibit a marked reduction in the number of cells (Figures 14, 16, 18, 44, 46, 48), but some alterations were evident. These changes were most evident in animals treated for 21 days and consisted of a slight reduction in the number of medullary lymphocytes with evidence of medullary epithelial cell degeneration (Figure 48). Thymic corpuscles were similar to those of control animals.

Light Microscopy - Study IV

Tissues for microscopic evaluation were obtained from DNCB sensitized animals in groups VI, VII, VIII, and IX. Sensitized animals were skin tested at time intervals of 0, 7, 14, or 21 days post-sensitization and the thymus collected immediately after the 72 hour reading.

Control animals. The thymus of control animals which were skin tested upon completion of DNCB sensitization (0 time interval) exhibited a marked increase in the relative proportion of small cortical lymphocytes (Figure 39). At low magnification (Figure 19), the cortex appeared to

exhibit a reduction in the number of lymphocytes but the apparent reduction in number was caused by the reduction in average cell size rather than to actual cell loss. The increase in small lymphocyte population at time interval 0 is particularly striking when the sensitized control animals of this group (VI-B) (Figure 39) are compared to the non-sensitized control animals in Study III (Figures 33, 35, 37). The relative number of small cortical lymphocytes decreased as the time interval between skin test and last day of sensitization increased. The changes between the 0 and the 21 day time intervals are illustrated in Figures 39 and 41.

The medulla and corticomedullary junctional areas exhibited no detectable changes.

Experimental animals. PH treated animals with cortical lymphocyte depletion showed evidence of lymphocyte replenishment even when examined immediately after completion of DNCB sensitization (0 time interval) (Figures 20, 30, 40). These experimental animals (VI-A) did not have the extensive cortical lymphocyte replenishment of the animals examined at the 21 day interval (IX-A) (Figures 20, 22, 30, 32, 40, 42). When the experimental animals in groups IX-A (21 day interval) were compared to the corresponding control group (IX-B), the experimental animals showed signs of overcompensation in cortical lymphocyte production (Figures 21, 22, 31, 32, 41, 42).

The thymic medullary regions of experimental animals examined at 0, 7, 14, and 21 day intervals were indistinguishable from control animals (Figures 49, 50, 51, 52).

Figure 13. Control Guinea Pig Thymus from Animal Treated with 7 Daily Injections of Water (Group III-B; H&E 230X).

Figure 14. Experimental Guinea Pig Thymus from Animal Treated with 7 Daily Injections of Procarbazine Hydrochloride (Group III-A; H&E 230X).

Figure 15. Control Guinea Pig Thymus from Animal Treated with 14 Daily Injections of Water (Group IV-B; H&E 230X).

Figure 16. Experimental Guinea Pig Thymus from Animal Treated with 14 Daily Injections of Procarbazine Hydrochloride (Group IV-A; H&E 230X).

Figure 17. Control Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Water (Group V-B; H&E 230X).

Figure 18. Experimental Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Procarbazine Hydrochloride (Group V-A; H&E 230X).

Figure 19. Control Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Water and Sacrificed at 0 Day Interval Post DNCB Sensitization (Group VI-B; H&E 230X).

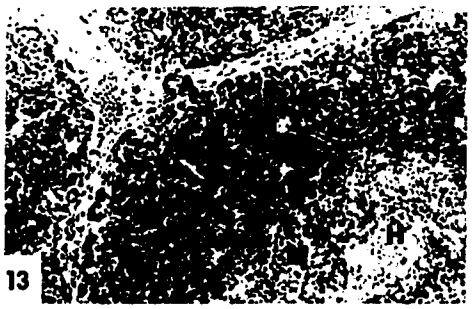
Figure 20. Experimental Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Procarbazine Hydrochloride and Sacrificed at 0 Day Interval Post DNCB Sensitization (Group VI-A; H&E 230X).

Figure 21. Control Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Water and Sacrificed at 21 Day Interval Post DNCB Sensitization (Group IX-B; H&E 230X).

Figure 22. Experimental Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Procarbazine Hydrochloride and Sacrificed at 21 Day Interval Post DNCB Sensitization (Group IX-A; H&E 230X).

LEGEND

C Cortex
CA Capsule (the arrows in Figures 14 and 16 indicate the plane of the connective tissue capsule between adjacent thymic lobules)
H Hassall's corpuscle
J Corticomedullary junction
M Medulla



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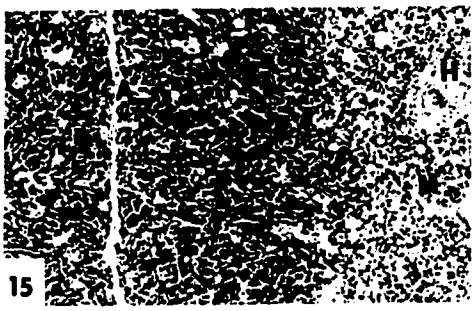

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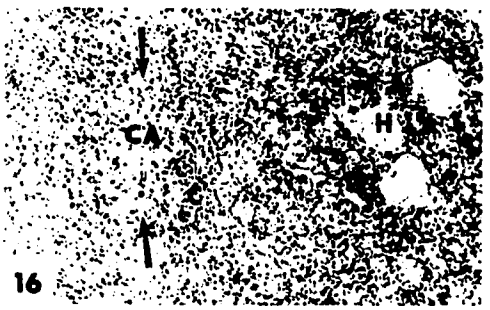

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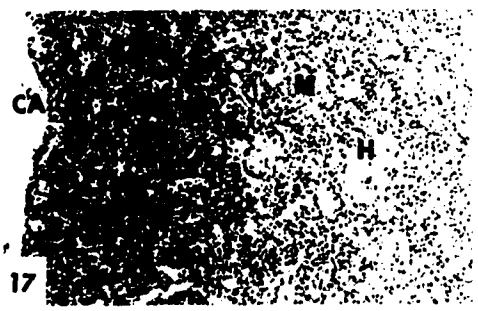

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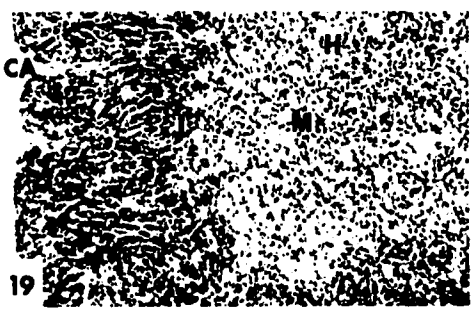

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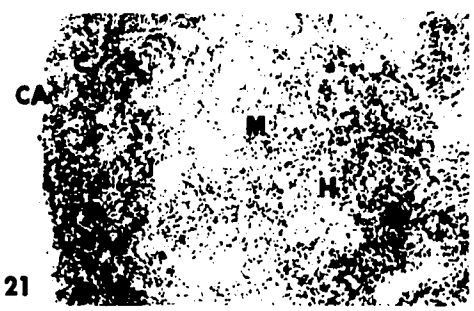

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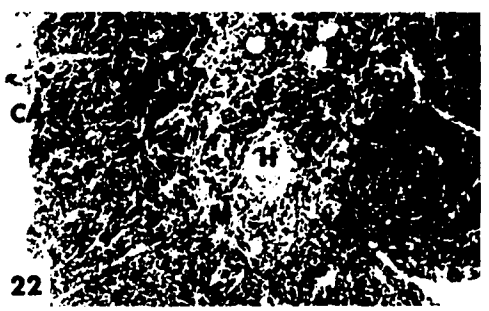

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Figure 23. Control Guinea Pig Thymus from Animal Treated with 7 Daily Injections of Water (Group III-B; H&E 920X).

Figure 24. Experimental Guinea Pig Thymus from Animal Treated with 7 Daily Injections of Procarbazine Hydrochloride (Group III-A; H&E 920X).

Figure 25. Control Guinea Pig Thymus from Animal Treated with 14 Daily Injections of Water (Group IV-B; H&E 920X).

Figure 26. Experimental Guinea Pig Thymus from Animal Treated with 14 Daily Injections of Procarbazine Hydrochloride (Group IV-A; H&E 920X).

Figure 27. Control Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Water (Group V-B; H&E 920X).

Figure 28. Experimental Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Procarbazine Hydrochloride (Group V-A; H&E 920X).

Figure 29. Control Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Water and Sacrificed at 0 Day Interval Post DNCB Sensitization (Group VI-B; H&E 920X).

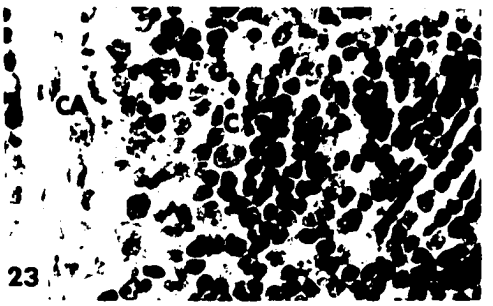
Figure 30. Experimental Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Procarbazine Hydrochloride and Sacrificed at 0 Day Interval Post DNCB Sensitization (Group VI-A; H&E 920X).

Figure 31: Control Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Water and Sacrificed at 21 Day Interval Post DNCB Sensitization (Group IX-B; H&E 920X).

Figure 32: Experimental Guinea Pig Thymus from Animal Treated with 21 Daily Injections of Procarbazine Hydrochloride and Sacrificed at 21 Day Interval Post DNCB Sensitization (Group IX-A; H&E 920X).

LEGEND

CA Capsule
C Cortex
H Hassall's corpuscle
J Corticomedullary junction
M Medulla



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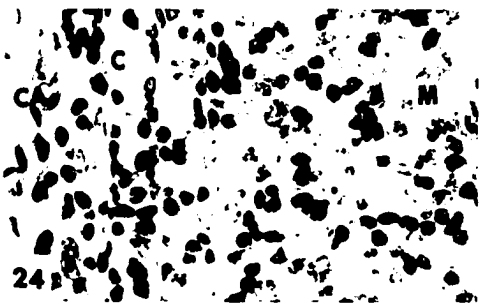

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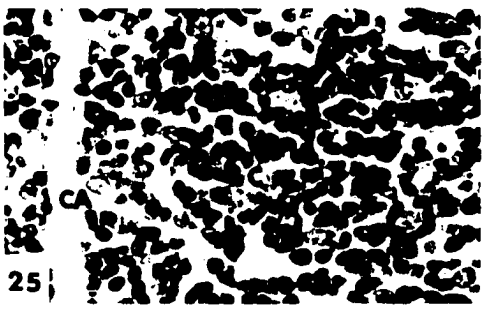

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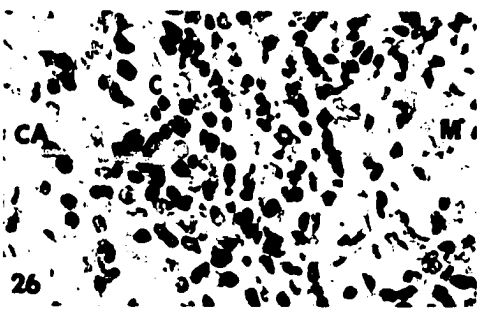

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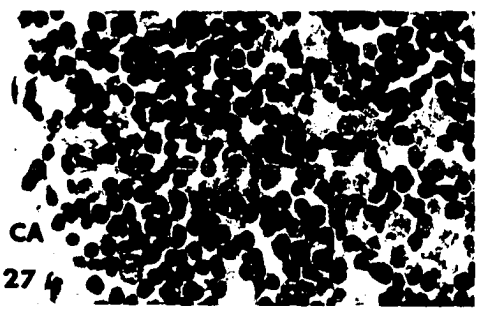

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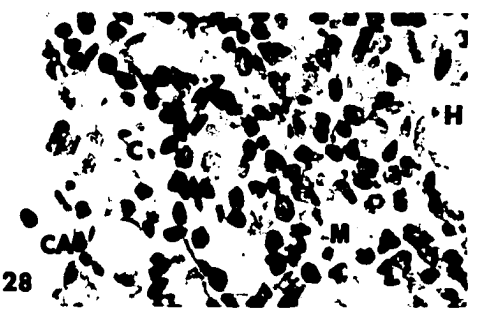

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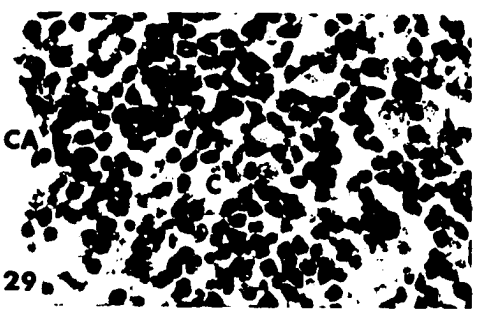

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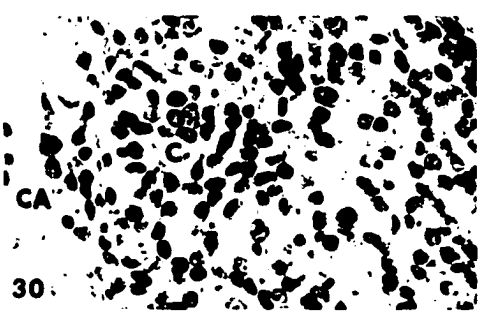

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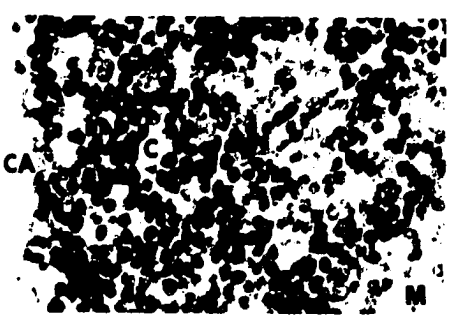

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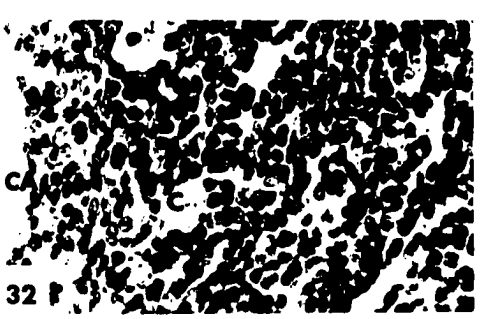

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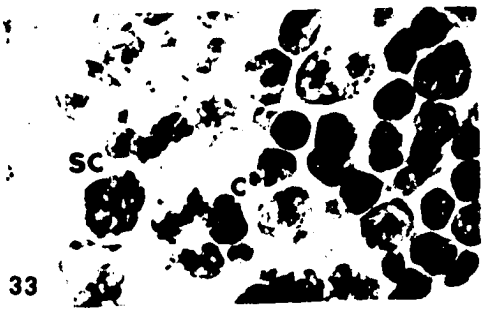
LEGEND

C Cortex
M Medulla
SC Subcapsular region of cortex



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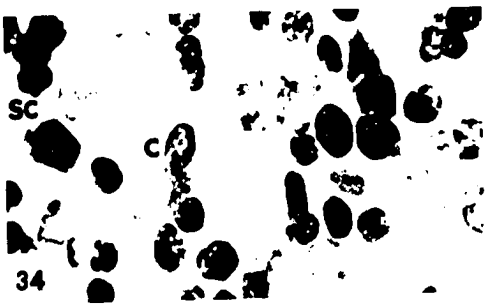

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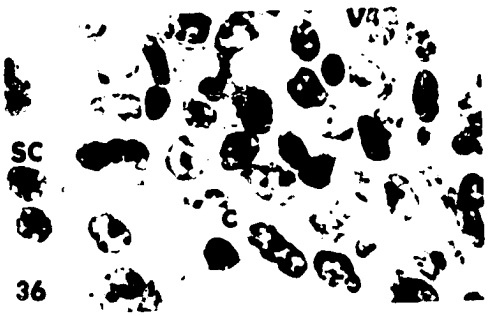

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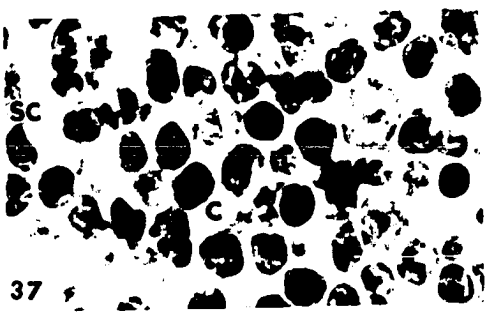

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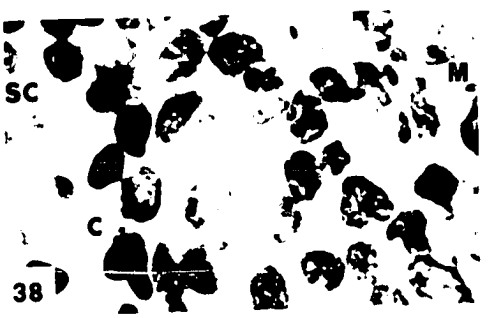

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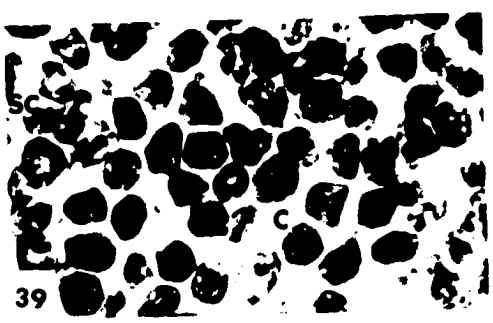

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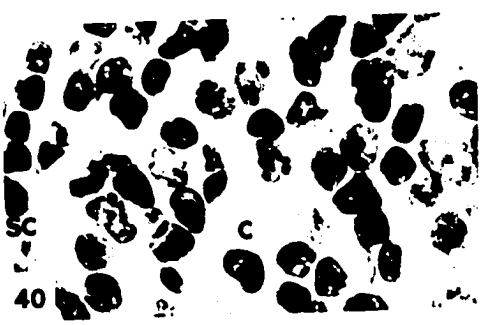

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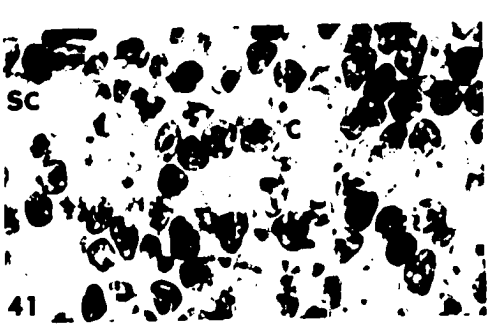

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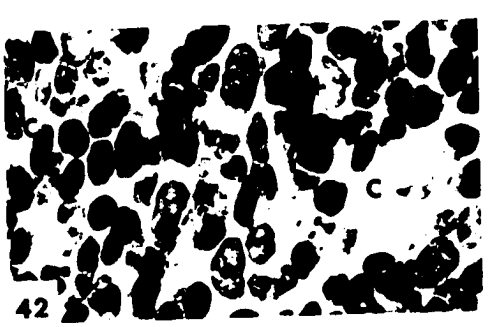

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Figure 43. Control Guinea Pig Thymic Medulla from Animal Treated with 7 Daily Injections of Water (Group III-B; H&E 2,310X).

Figure 44. Experimental Guinea Pig Thymic Medulla from Animal Treated with 7 Daily Injections of Procarbazine Hydrochloride (Group III-A; H&E 2,310X).

Figure 45. Control Guinea Pig Thymic Medulla from Animal Treated with 14 Daily Injections of Water (Group IV-B; H&E 2,310X).

Figure 46. Experimental Guinea Pig Thymic Medulla from Animal Treated with 14 Daily Injections of Procarbazine Hydrochloride (Group IV-A; H&E 2,310X).

Figure 47. Control Guinea Pig Thymic Medulla from Animal Treated with 21 Daily Injections of Water (Group V-B; H&E 2,310X).

Figure 48. Experimental Guinea Pig Thymic Medulla from Animal Treated with 21 Daily Injections of Procarbazine Hydrochloride (Group V-A; H&E 2,310X).

Figure 49. Control Guinea Pig Thymic Medulla from Animal Treated with 21 Daily Injections of Water and Sacrificed at 0 Day Interval Post DNCB Sensitization (Group VI-B; H&E 2,310X).

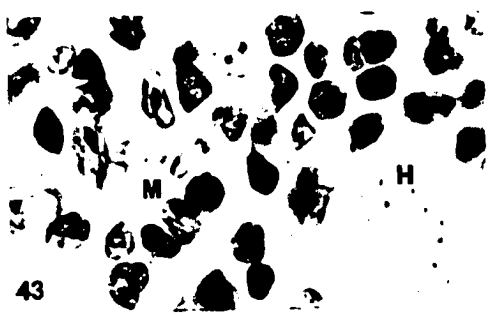
Figure 50. Experimental Guinea Pig Thymic Medulla from Animal Treated with 21 Daily Injections of Procarbazine Hydrochloride and Sacrificed at 0 Day Interval Post DNCB Sensitization (Group VI-A; H&E 2,310X).

Figure 51. Control Guinea Pig Thymic Medulla from Animal Treated with 21 Daily Injections of Water and Sacrificed at 21 Day Interval Post DNCB Sensitization (Group IX-B; H&E 2,310X).

Figure 52. Experimental Guinea Pig Thymic Medulla from Animal Treated with 21 Daily Injections of Procarbazine Hydrochloride and Sacrificed at 21 Day Interval Post DNCB Sensitization (Group IX-A; H&E 2,310X).

LEGEND

H Hassall's corpuscle
M Medulla



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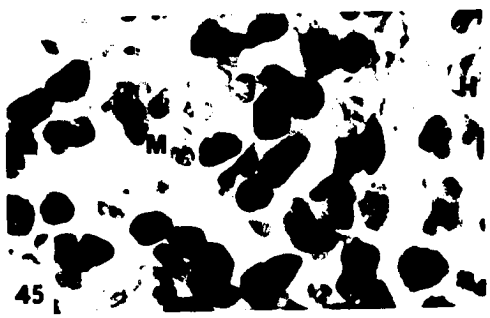

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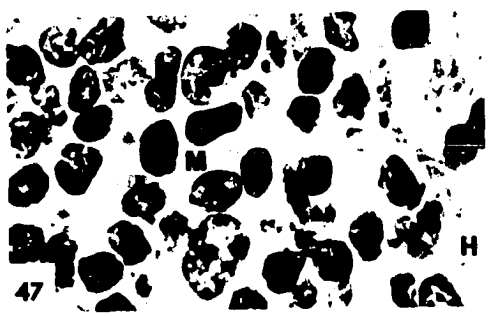

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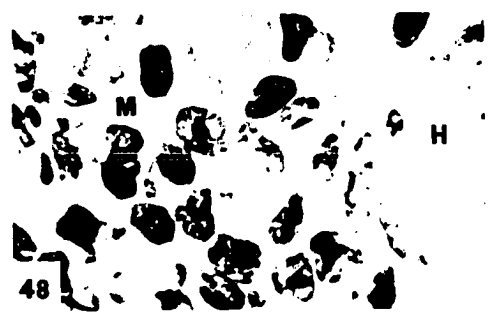

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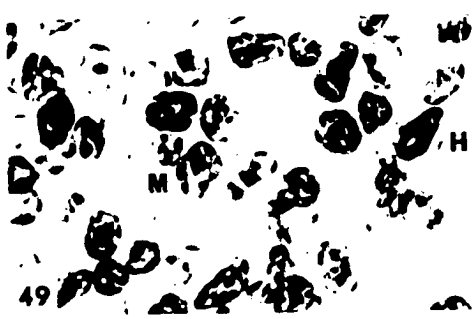

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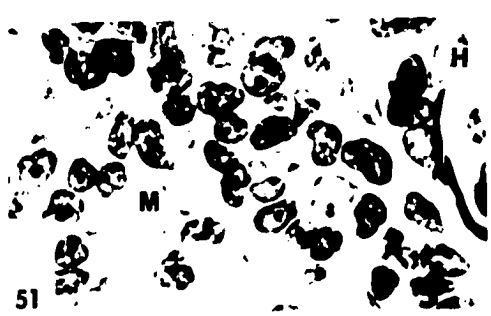

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Electron Microscopy - Study III

Tissue for electron microscopic evaluation was obtained from animals in groups III, IV and V. These animals received PH or water for 7, 14, or 21 days prior to sacrifice.

Control animals. The thymic cortex of control animals receiving 7, 14, or 21 daily injections of water showed stellate epithelial cells surrounded by small, medium and large sized thymic lymphocytes (Figures 53, 54, 55). The lymphocyte population consisted of cells in various stages of differentiation and contained an impressive proportion of medium and large sized cortical lymphocytes (Figures 53, 54, 55). Many lymphocytes demonstrated short blunt cytoplasmic processes which were interdigitated with the perinuclear cytoplasm or with the processes of adjacent epithelial cells (Figures 53, 54).

Cortical thymic epithelial cells of control animals demonstrated the classic large vesicular nucleus with prominent nucleolus and sparse perinuclear cytoplasm (Figures 53, 54). The epithelial cell cytoplasm and peripheral cytoplasmic processes contained tonofibrils, electron dense bodies and membrane bound bodies (Figures 53, 54, 55). Cytoplasmic processes of adjacent cortical epithelial cells were joined by typical desmosomes (Figure 55).

Medullary regions of control animals displayed a normal arrangement of thymic lymphocytes intermingled with medullary epithelial cell processes (Figure 56). This region also displayed normal "medullary squamous epithelial cells" forming thymic corpuscles (Hassall's corpuscles; Figure 57) as well as the more peripherally located "cystic medullary epithelial" cells (Figure 56).

Experimental animals. The thymic cortex of animals receiving 7, 14 or 21 daily injections of PH were markedly depleted of small, medium and large sized lymphocytes (Figures 58, 59, 60, 61, 62, 63). After short term procarbazine treatment (7 days), the residual lymphocytes demonstrated nuclear chromatin patterns which were similar to those of control animals (Figures 53, 54, 55, 58, 59, 60, 61, 62, 63). With longer treatment (14 or 21 days), the cortical thymic lymphocytes exhibited varying degrees of cell injury, but overtly necrotic lymphocytes were not seen. Specific alterations in the residual lymphocyte population included nuclear polymorphism (Figures 60, 61) and cytoplasmic hypertrophy (Figures 60 and 61). The damaged lymphocytes exhibited morphological evidence of phagocytosis with the accumulation of intracytoplasmic debris (lamellar and amorphous electron dense material; Figures 58, 60). Tightly interlocked cytoplasmic processes (Figure 59) were occasionally demonstrated between the residual cortical lymphocytes.

Cortical epithelial cells of animals receiving PH were highly altered. These cellular changes appeared with short exposure (7 days) and became more severe with prolonged treatment. The nuclear changes were less dramatic than cytoplasmic changes and consisted of a progressive polymorphism in nuclear morphology without significant changes in the nuclear chromatin pattern (Figures 58, 60, 61, 62). The cytoplasmic regions of cortical epithelial cells from animals treated with 7, 14 or 21 injections of PH demonstrated marked degrees of cell injury and cytoplasmic degeneration. The perinuclear cytoplasm and peripheral cytoplasmic processes were greatly enlarged and contained an increased number of intracytoplasmic structures (Figures 58, 60, 61, 62, 63). These cytoplasmic

structures consisted of dense and membrane bound bodies and vacuoles with and without granular material (Figures 58, 59, 60, 61, 62, 63). The epithelial cells of animals treated with 14 daily injections of PH displayed the most severe degree of cellular injury (Figures 60, 61, 62) and exhibited polymorphonuclear leukocyte infiltration. These infiltrating cells were found within the cytoplasm of damaged epithelial cells (Figures 61, 62). After 21 daily injections of PH, macrophages could also be demonstrated within the thymus cortex (Figure 63). Macrophages were located within the subcapsular areas and displayed evidence of intracytoplasmic lysosomal-like bodies but macrophages were never demonstrated inside the damaged epithelial cells.

Animals treated with 7 daily injections of PH demonstrated slight ultrastructural alteration of their medullary lymphocytes (Figures 56, 57, 64, 65). The medullary lymphocytes in animals treated with PH for 14 days demonstrated morphological alterations similar to those noted in the cortex (Figures 60, 61, 62, 66).

The medullary epithelial cells in animals treated with 7 daily injections of PH demonstrated minor degrees of nuclear and cytoplasmic alteration (Figures 64, 65). Changes in the nuclear architecture and chromatin pattern of medullary epithelial cells were unremarkable (Figures 64, 65) but the cytoplasmic regions displayed increased amounts of rough endoplasmic reticulum and lipid vacuoles (Figure 64). The thymic medullary epithelial cells in animals treated with 14 daily injections of PH demonstrated morphological alterations similar to those noted in the cortex (Figures 60, 61, 62, 66).

Electron Microscopy - Study IV

Tissue for electron microscopic evaluation was obtained from DNCB sensitized experimental and control animals in groups VI, VII, VIII and IX. Sensitized animals were skin tested at time intervals of 0, 7, 14 or 21 days post-sensitization and the thymus collected immediately after the 72 hour reading.

Control animals. Ultrastructural examination of thymus tissues from control animals sacrificed at 0 and 21 day intervals post-sensitization demonstrated a predominance of small cortical lymphocytes (Figures 67, 68). The electron microscopic characteristics of the cortical lymphocytes and epithelial cells were similar to those listed for control animals in Study III (Figures 53, 54, 55, 67, 68).

The ultrastructural characteristics of the medullary lymphocytes and epithelial cells were similar to those listed for control animals in Study III.

Experimental animals. Electron microscopic examination of cortical thymic lymphocytes from PH treated animals sacrificed at 0 and 21 day intervals after completion of DNCB sensitization revealed a mixture of small, medium and large sized lymphocytes (Figures 69, 70). Although there was a slight predominance of medium and large sized cells, the nuclear and cytoplasmic characteristics of these lymphocytes were unremarkable.

Thymic cortical epithelial cells from animals sacrificed at 0 day interval post-sensitization were similar in ultrastructural changes to those of animals in Study III treated with either 14 or 21 daily injections of PH (Figures 60, 61, 62, 69). Cortical epithelial cells in

animals sacrificed 21 days post-sensitization were indistinguishable from those of corresponding control groups (Figures 68, 70).

Thymic medullary lymphocytes, epithelial cells and thymic corpuscles (Hassall's corpuscles) were unaltered morphologically.

Figure 53. Control Animal, Study III: Thymic cortex of guinea pig treated with 14 daily injections of water. The cortical epithelial cells (E) exhibit a large vesicular nucleus (N) with a delicate layer of marginal chromatin and a prominent nucleolus. The epithelial cells have a thin rim of electron-lucent perinuclear cytoplasm with long processes (P) which extend into the surrounding population of thymic lymphocytes. The lymphocyte population consists of cells in various stages of differentiation and consists primarily of small (S) and medium (M) sized thymic lymphocytes. (uranyl acetate and lead citrate 6,670X).

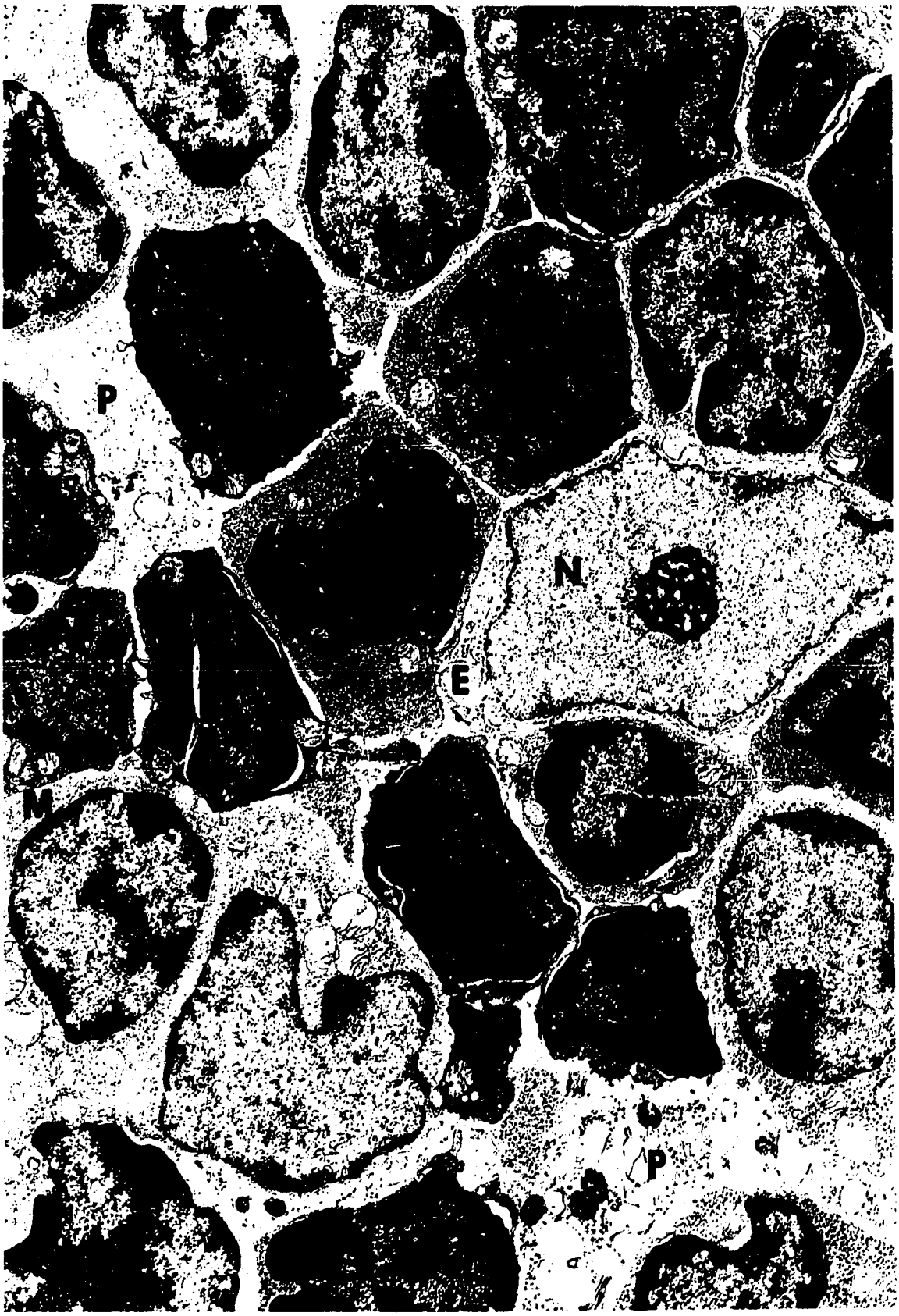

P
N
E
M
P

Figure 54. Control Animal, Study III: Thymic cortex of guinea pig treated with 14 daily injections of water. The thymus cortex contains epithelial cells (E) surrounded by a mixture of small (S), medium (M) and large (L) sized lymphocytes. The perinuclear cytoplasm of the cortical epithelial cell shows two short blunt cytoplasmic processes (*) extending from the adjacent large lymphocyte. (uranyl acetate and lead citrate 6,900X).

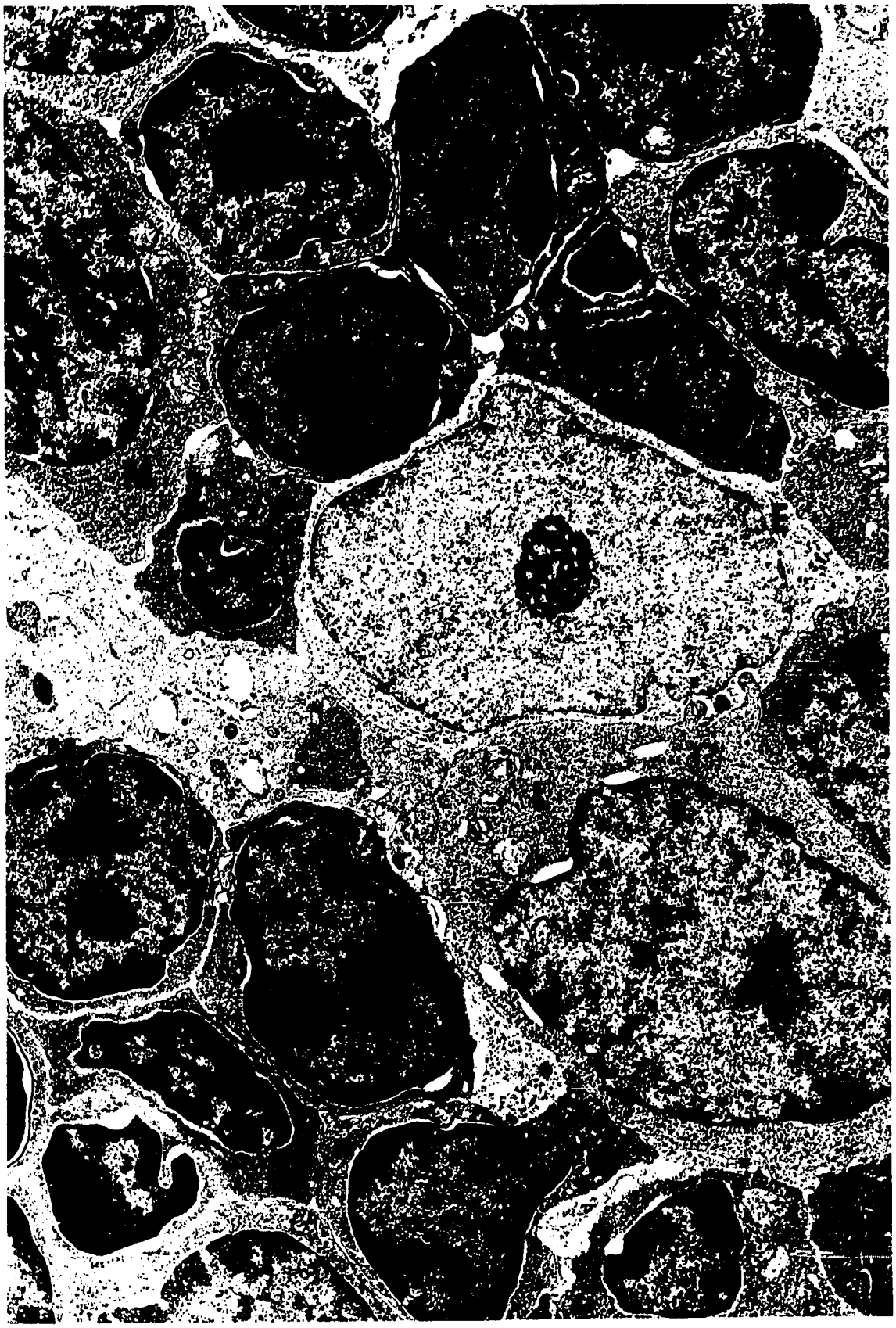


Figure 55. Control Animal, Study III: Thymic cortex of guinea pig treated with 14 daily injections of water. The cortical thymic epithelial cell processes (P) contain a few cytoplasmic structures, e.g., electron dense bodies (→), membrane bound bodies (MB), and are attached to the peripheral processes of adjacent epithelial cells by typical desmosomes (D). The normal population of small (S), medium (M) and large (L) sized thymic lymphocytes are easily detected between the peripheral processes of the cortical epithelial cells. (uranyl acetate and lead citrate 7,200X).

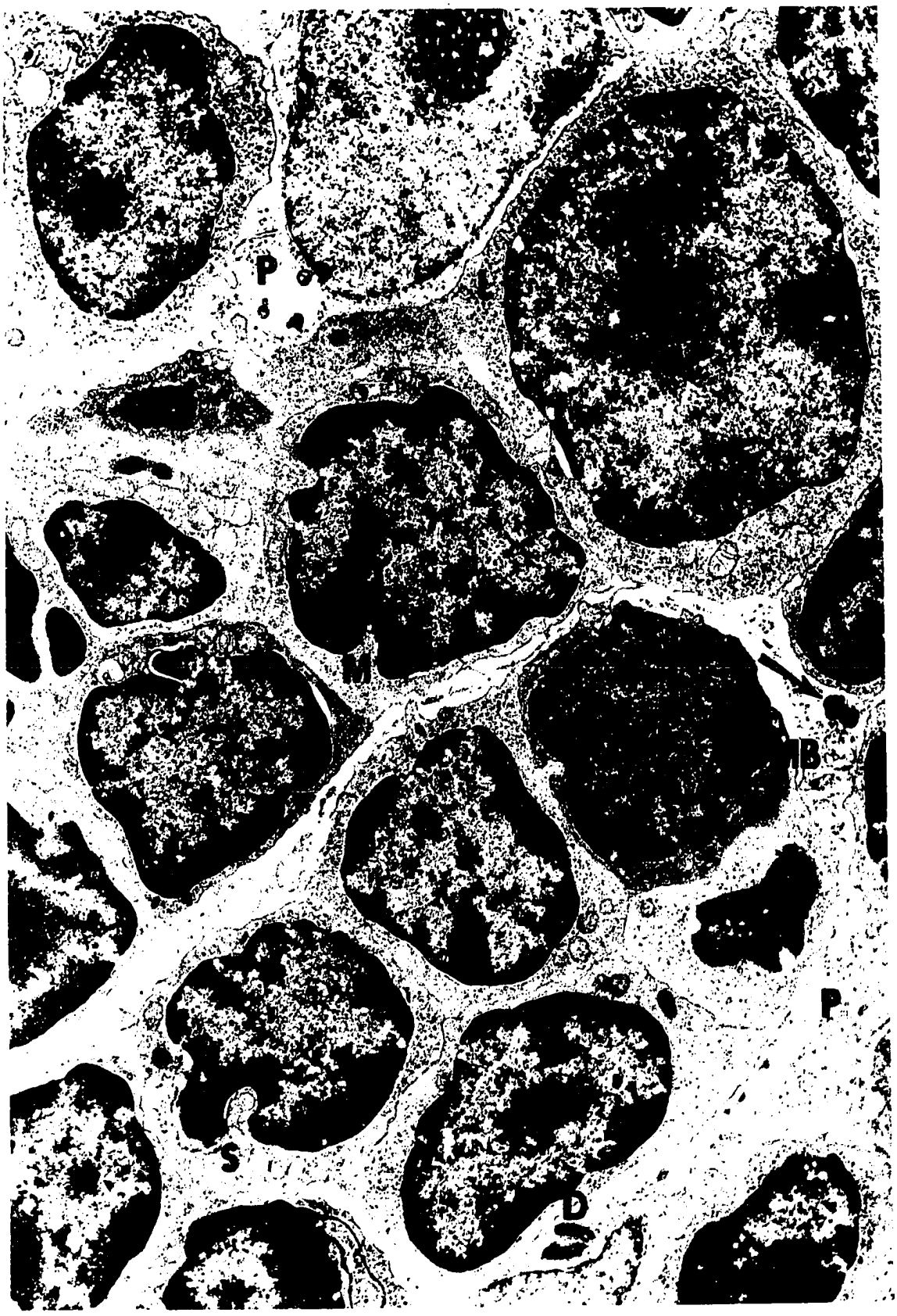

P
P
S
D

Figure 56. Control Animal, Study III; Thymic medulla of guinea pig treated with 14 daily injections of water. A cystic medullary epithelial cell (E) and a typical population of medullary lymphocytes are shown. Note the difference in cytoplasmic density of the medullary lymphocyte population (L1 and L2). (uranyl acetate and lead citrate 5,520X).

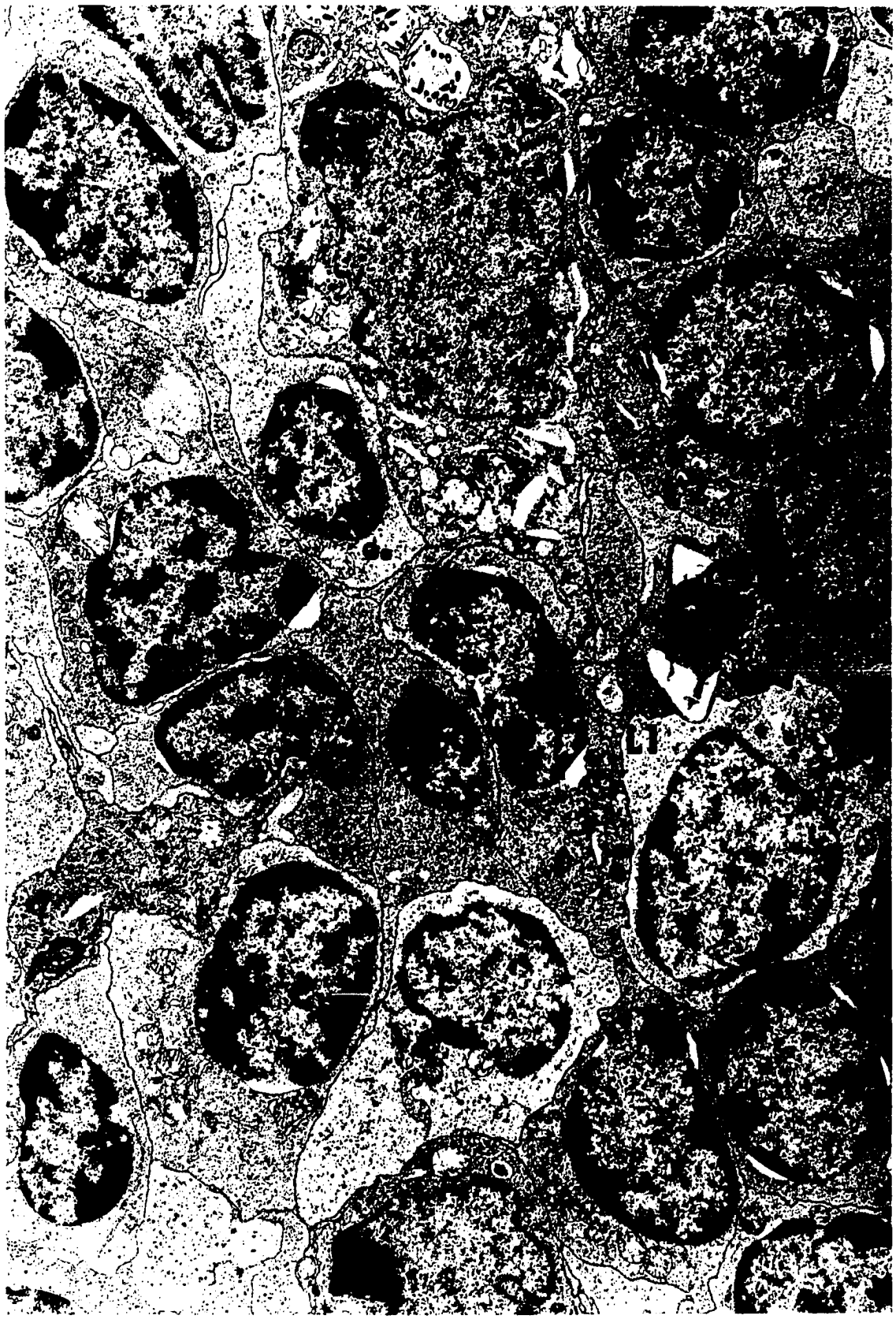

L1

Figure 57. Control Animals, Study III: Thymic medulla of guinea pig treated with 14 daily injections of water. The medullary region exhibits a Hassall's corpuscle (H) containing intra-corpuscular debris (D) surrounded by squamous epithelial cells (S). A representative population of medullary lymphocytes (L) appear at the periphery of the Hassall's corpuscle. (uranyl acetate and lead citrate 5,750X).

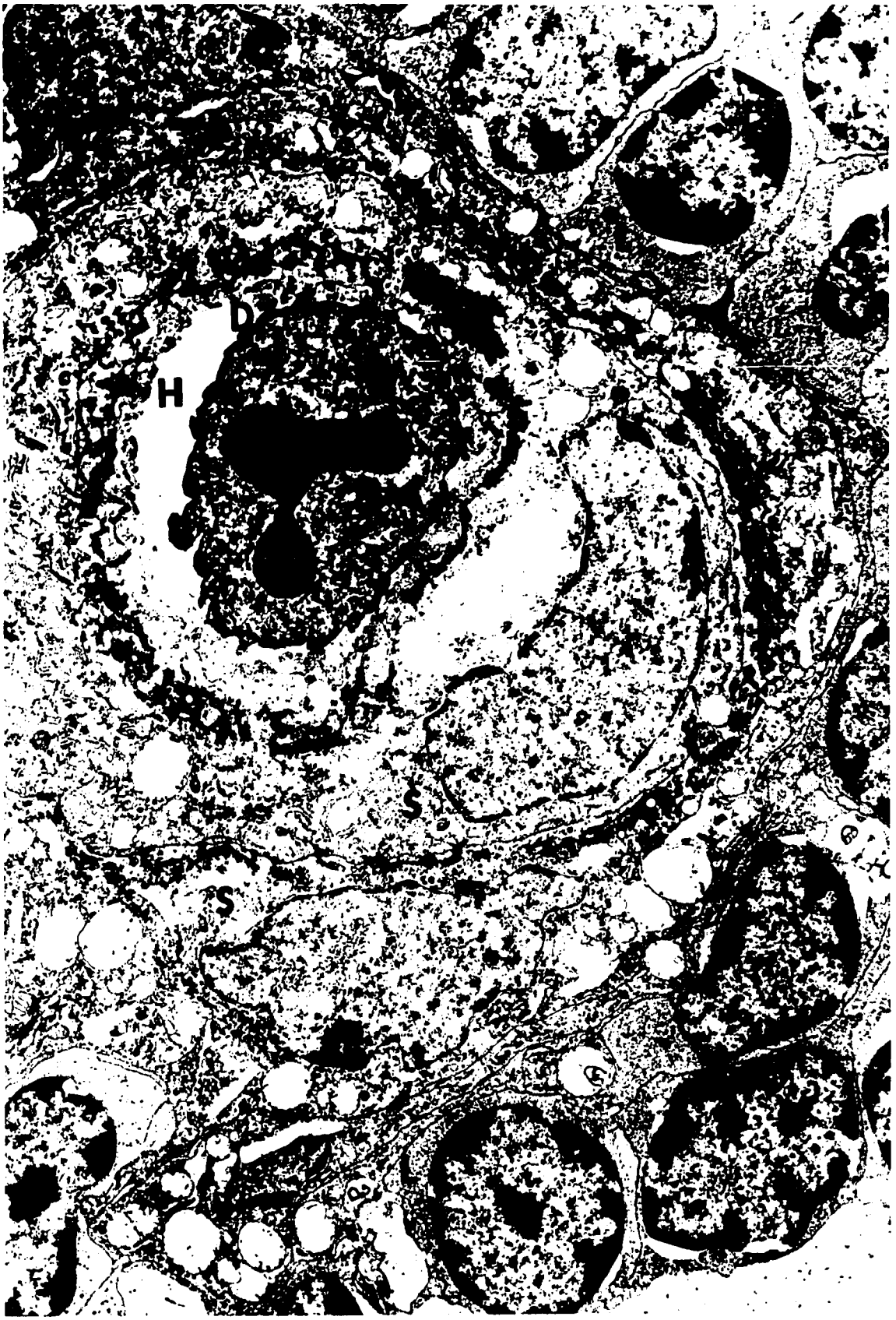

D
H
S
S

Figure 58. Experimental Animal, Study III: Thymic cortex of guinea pig treated with 7 daily injections of procarbazine hydrochloride. The cortex exhibits a marked depletion of thymic lymphocytes. The residual population consists of only a few small (S) and medium (M) sized cortical lymphocytes. A damaged medium sized lymphocyte exhibits morphological evidence suggesting phagocytosis of lamellar debris (LD). Cortical epithelial cells (E) demonstrate evidence of degeneration. Perinuclear cytoplasm and cytoplasmic processes of the epithelial cells are greatly enlarged, exhibit tonofilaments (→) and contain increased numbers of electron dense bodies (ED) and membrane bound bodies (MB). (uranyl acetate and lead citrate 7,500X).

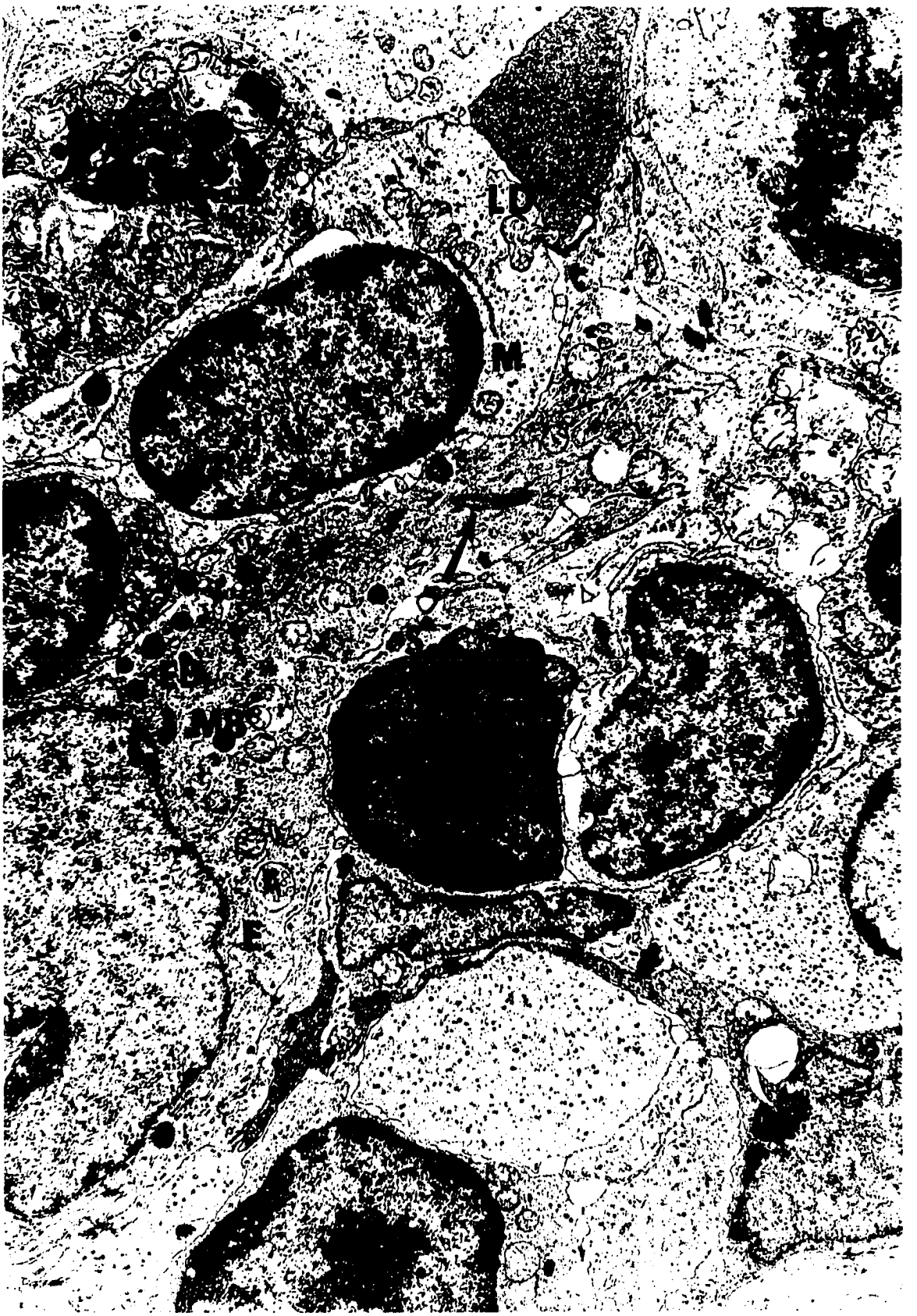

LD
M
MB
E

Figure 59. Experimental Animal, Study III: Thymic cortex of guinea pig treated with 7 daily injections of procarbazine hydrochloride. In other areas, the cortex exhibits a depletion of thymic lymphocytes, but this area of the depleted cortex shows a representative group of the residual lymphocyte population (L). An interlocking cytoplasmic process (→) can be seen between two of the residual lymphocytes. Epithelial cell processes (C) are enlarged and contain increased numbers of abnormal structures. (uranyl acetate and lead citrate 8,100X).

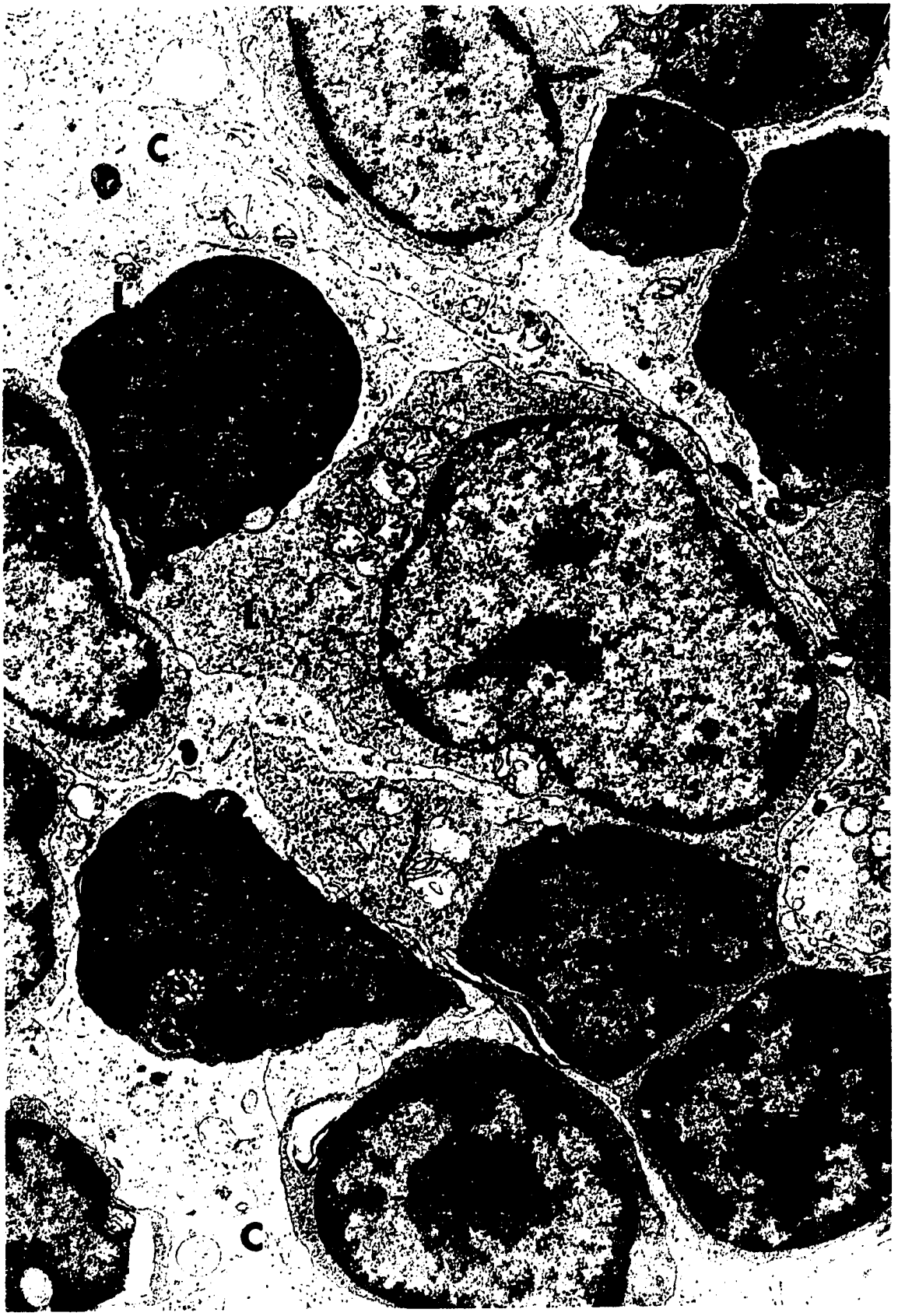

C
C

Figure 60. Experimental Animal, Study III: Thymic cortex of guinea pig treated with 14 daily injections of procarbazine hydrochloride. The cortex exhibits marked cellular depletion. Residual cortical lymphocytes exhibit varying degrees of cell injury but overtly necrotic cells are not seen. Specific alterations include slight nuclear changes and the accumulation of intracytoplasmic amorphous debris (→) which suggests phagocytic activity. The cortical epithelial cells (E) display evidence of degeneration. Degenerative changes consist of cytoplasmic hypertrophy with the appearance of increased numbers of abnormal structures, e.g., membrane bound bodies (MG), electron dense bodies (ED) and cystic vacuoles with or without granular material (C). (uranyl acetate and lead citrate 6,210X).

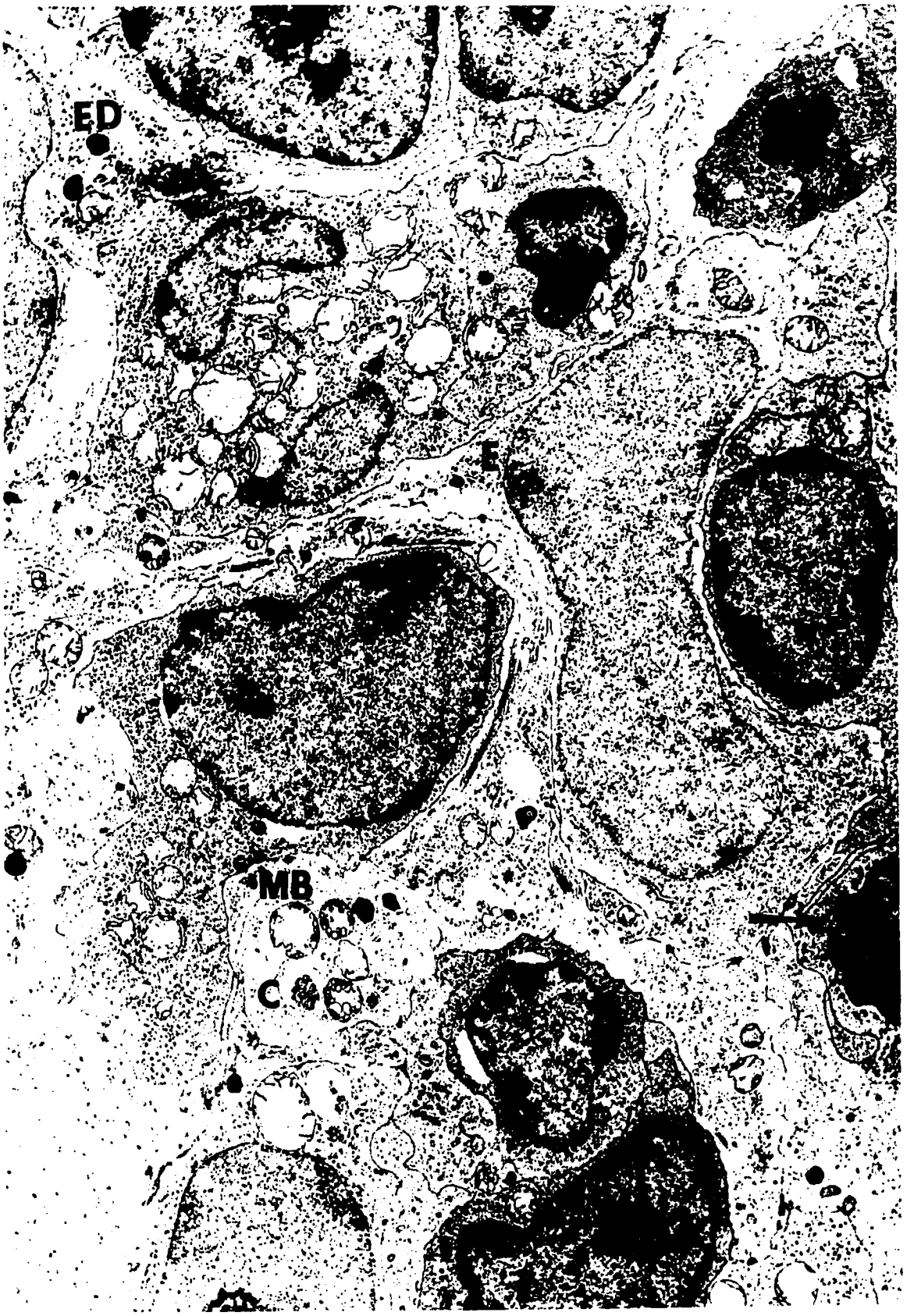

ED
MB
C

Figure 61. Experimental Animal, Study III: Thymic cortex of guinea pig treated with 14 daily injections of procarbazine hydrochloride. The thymic cortex exhibits evidence of marked lymphocytic depletion. The few remaining lymphocytes (L) demonstrate nuclear polymorphism (P) and cytoplasmic expansion (C). The cortical epithelial cells (E) exhibit less dramatic alterations in nuclear morphology than the residual lymphocytes. The cytoplasmic regions of the cortical epithelial cells show marked degenerative changes. This micrograph shows a polymorphonuclear leukocyte (G) located within the degenerating cytoplasm of a cortical epithelial cell (E1). (uranyl acetate and lead citrate 5,520X).

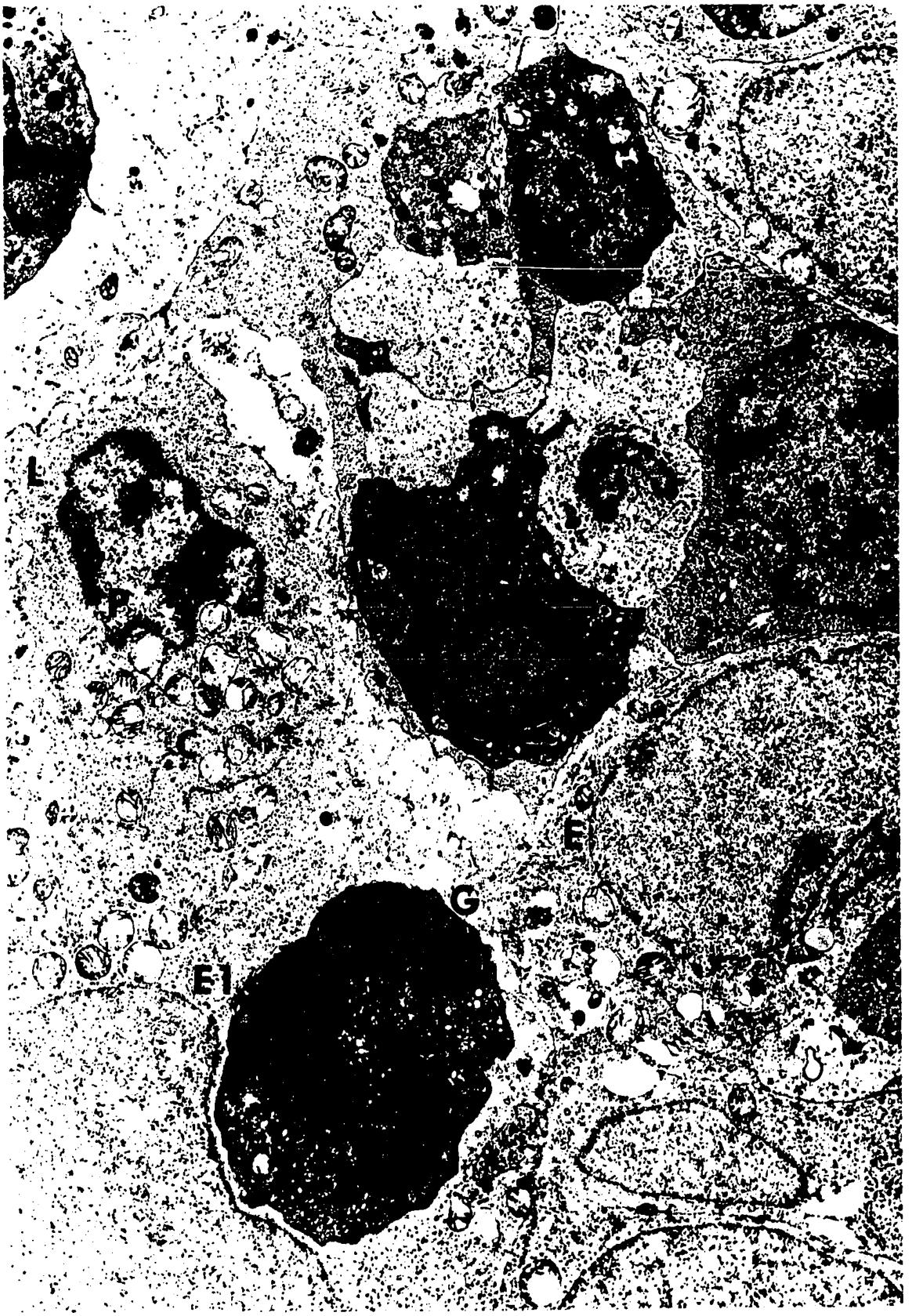

L
E1
G
E1

Figure 62. Experimental Animal, Study III: Thymic cortex of guinea pig treated with 14 daily injections of procarbazine hydrochloride. The cortex exhibits marked lymphocytic depletion and severe derangement. The few residual lymphocytes (L) demonstrate evidence of cellular damage. The cortical epithelial cells (E) demonstrate nuclear polymorphism and contain increased numbers of electron dense bodies (ED), membrane bound bodies (MB) and vesicular cysts. Polymorphonuclear leukocytes (G) are seen within the degenerating epithelial cell cytoplasm. (uranyl acetate and lead citrate 5,750X).

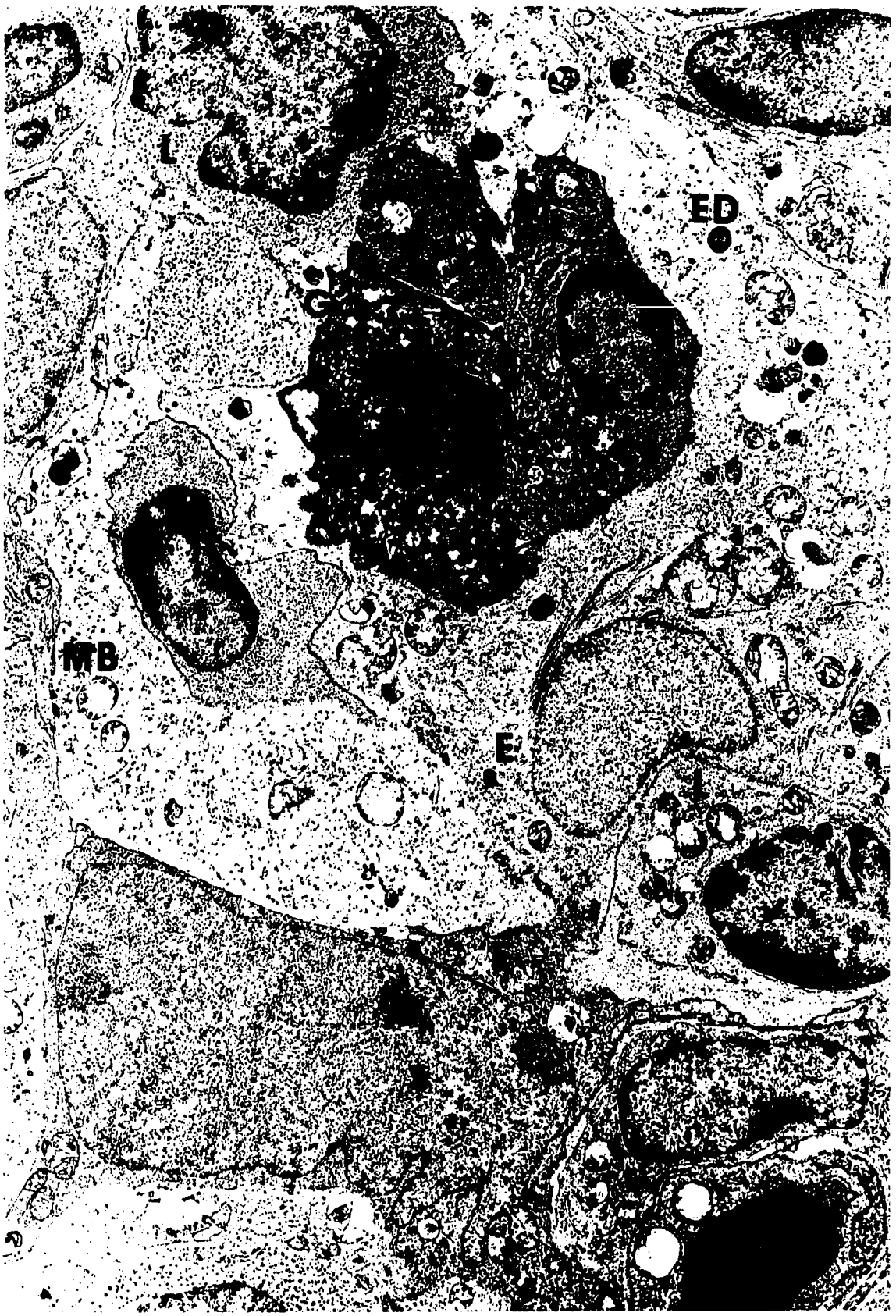

L
ED
G
MB
E

Figure 63. Experimental Animal, Study III: Thymic subcapsular region of guinea pig treated with 21 daily injections of procarbazine hydrochloride. The subcapsular region shows marked depletion of cortical lymphocytes. The remaining lymphocytes (L) demonstrate increased nuclear density. The epithelial cell (E) cytoplasmic processes (P) are greatly expanded and contain electron dense bodies (ED), membrane bound bodies (MB), lipid vacuoles (8), tonofilaments (→) and are connected to adjacent epithelial cells by desmosomes (D). The macrophages (M) demonstrate an abundance of lysosomal-like bodies. (uranyl acetate and lead citrate 6,440X).

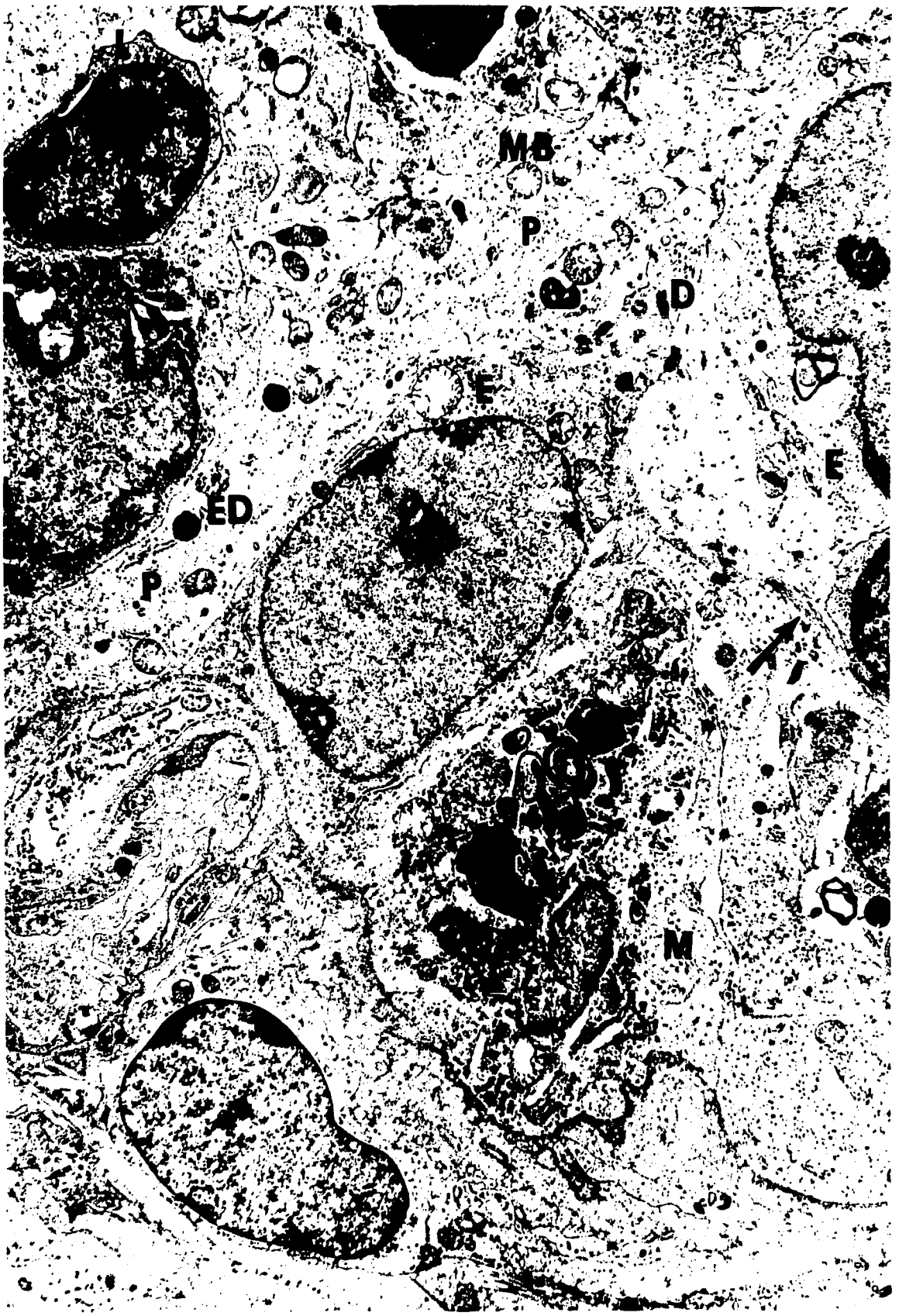

MB
P
D
E
ED
P
E
M

Figure 64. Experimental Animal, Study III: Thymic medulla of guinea pig treated with 7 daily injections of procarbazine hydrochloride. The medullary lymphocytes (L) exhibit slight morphological alteration. One medullary epithelial cell (E) shows an abundance of rough endoplasmic reticulum (ER) with distended mitochondria (M) and lipid vacuoles (*). This micrograph shows increased intercellular spaces and the presence of a polymorphonuclear leukocyte (G) in the cleft (H) of the Hassall's corpuscle. (uranyl acetate and lead citrate 7,200X).

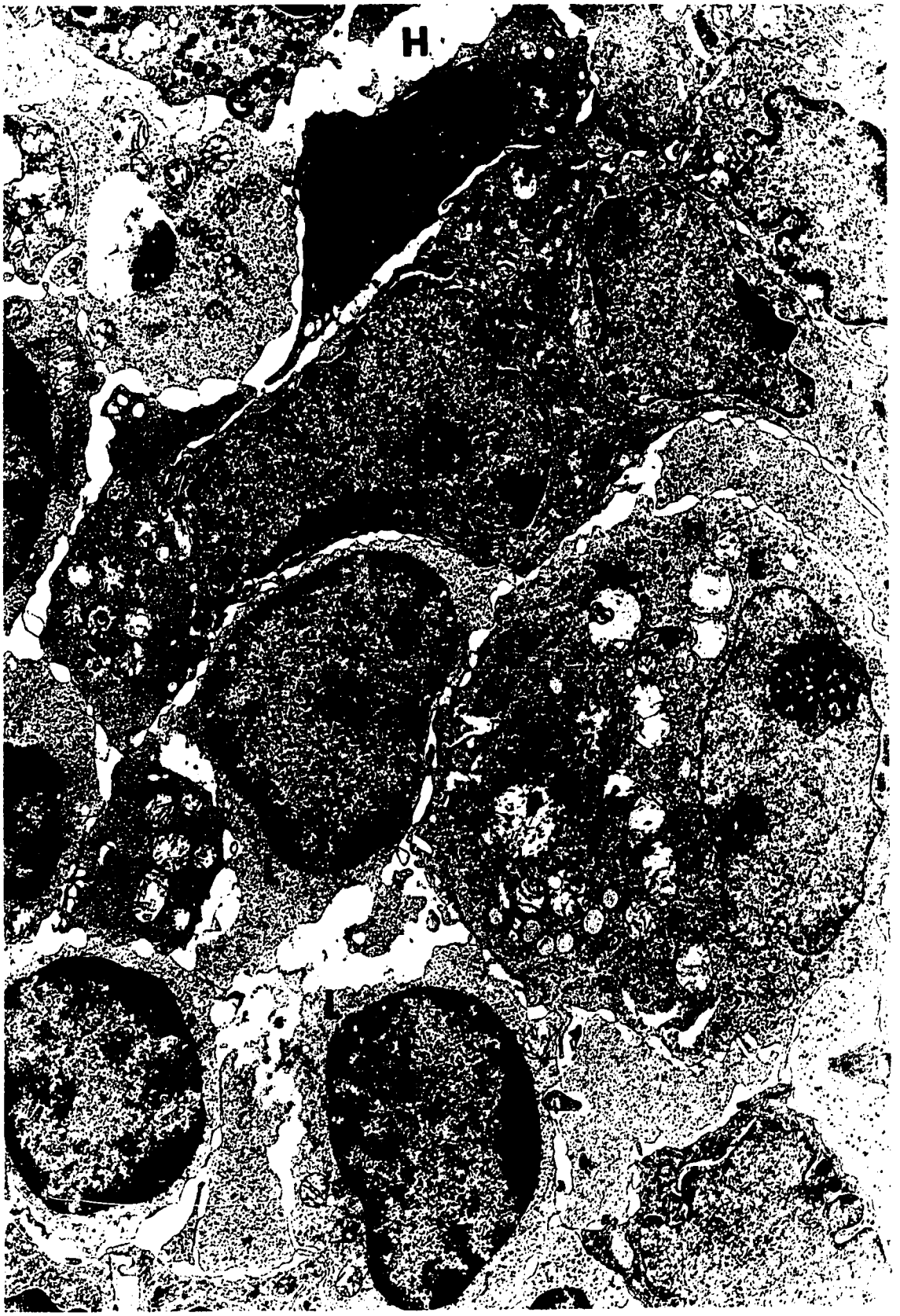

H

Figure 65. Experimental Animal, Study III: Thymic medulla of guinea pig treated with 7 daily injections of procarbazine hydrochloride. The medullary lymphocytes (L) do not show signs of morphological alteration. Villous medullar epithelial cells (E) display numerous microvilli projecting into the adjacent cleft (H) of Hassall's corpuscle. The peripheral processes (P) of the medullary epithelial cells contain an abundance of small intracytoplasmic vesicles (→). (uranyl acetate and lead citrate 7,500X).


H

Figure 66. Experimental Animal, Study III: Thymic medulla of guinea pig treated with 14 daily injections of procarbazine hydrochloride. This micrograph demonstrates marked depletion of medullary lymphocytes. The remaining lymphocytes (L) are moderately altered and show signs of nuclear polymorphism. The medullary epithelial cell (E) cytoplasmic regions (C) are greatly enlarged but remain connected to adjacent epithelial cells by desmosomes (D). This representative area shows cellular debris (→) and the presence of polymorphonuclear leukocytes (G). (uranyl acetate and lead citrate 5,520X).

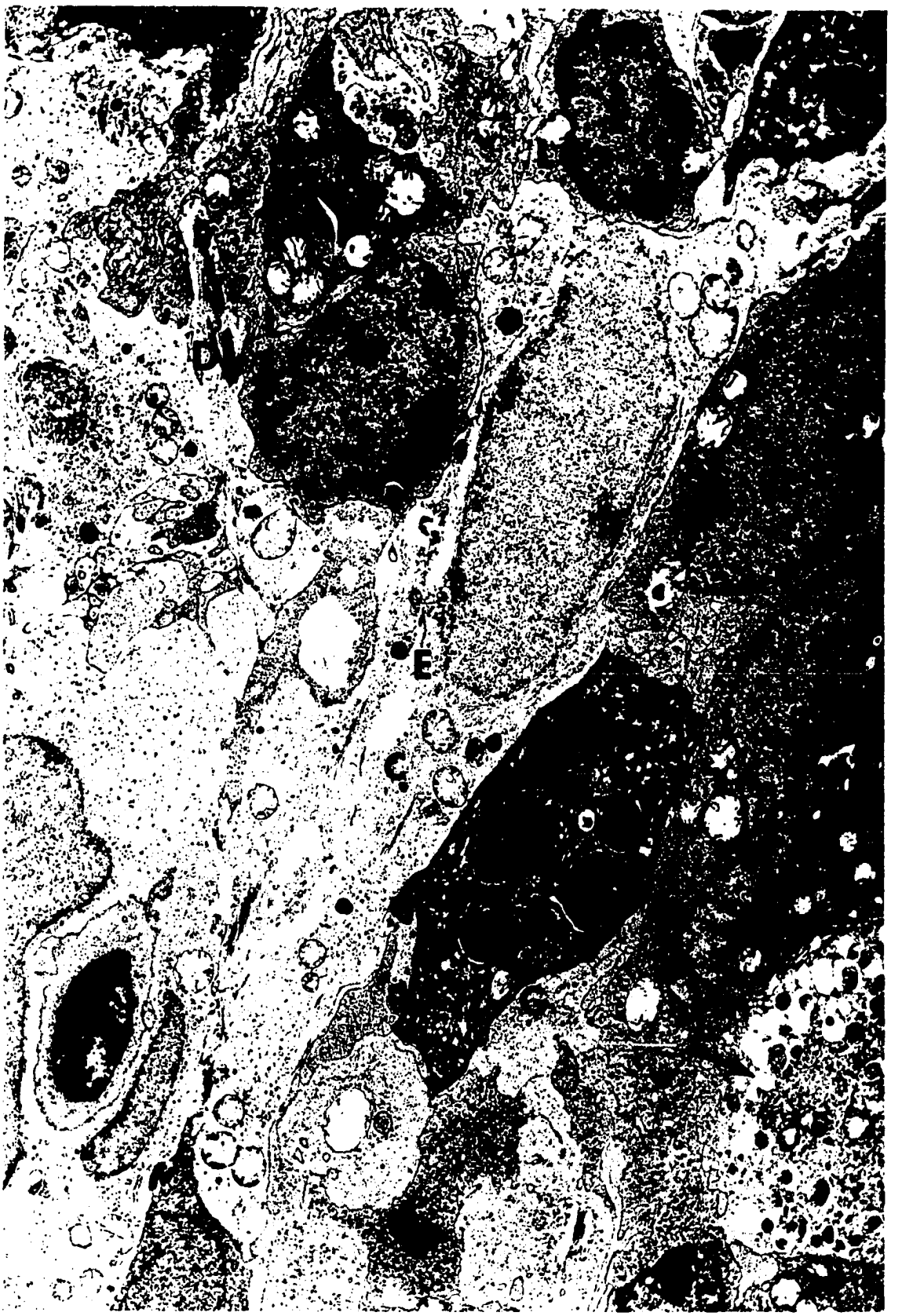

D
E

Figure 67. Control Animal, Study IV: Thymic cortex of control guinea pig sacrificed at 0 day interval post DNCB sensitization. This micrograph shows an abundance of small (S) and medium (M) sized cortical lymphocytes. The cortical epithelial cells (E) exhibit large vesicular nuclei (N) with long thin cytoplasmic processes (P). Processes are dispersed between adjacent lymphocytes and are connected to other epithelial cell processes by desmosomes (D). (uranyl acetate and lead citrate 6,440X).

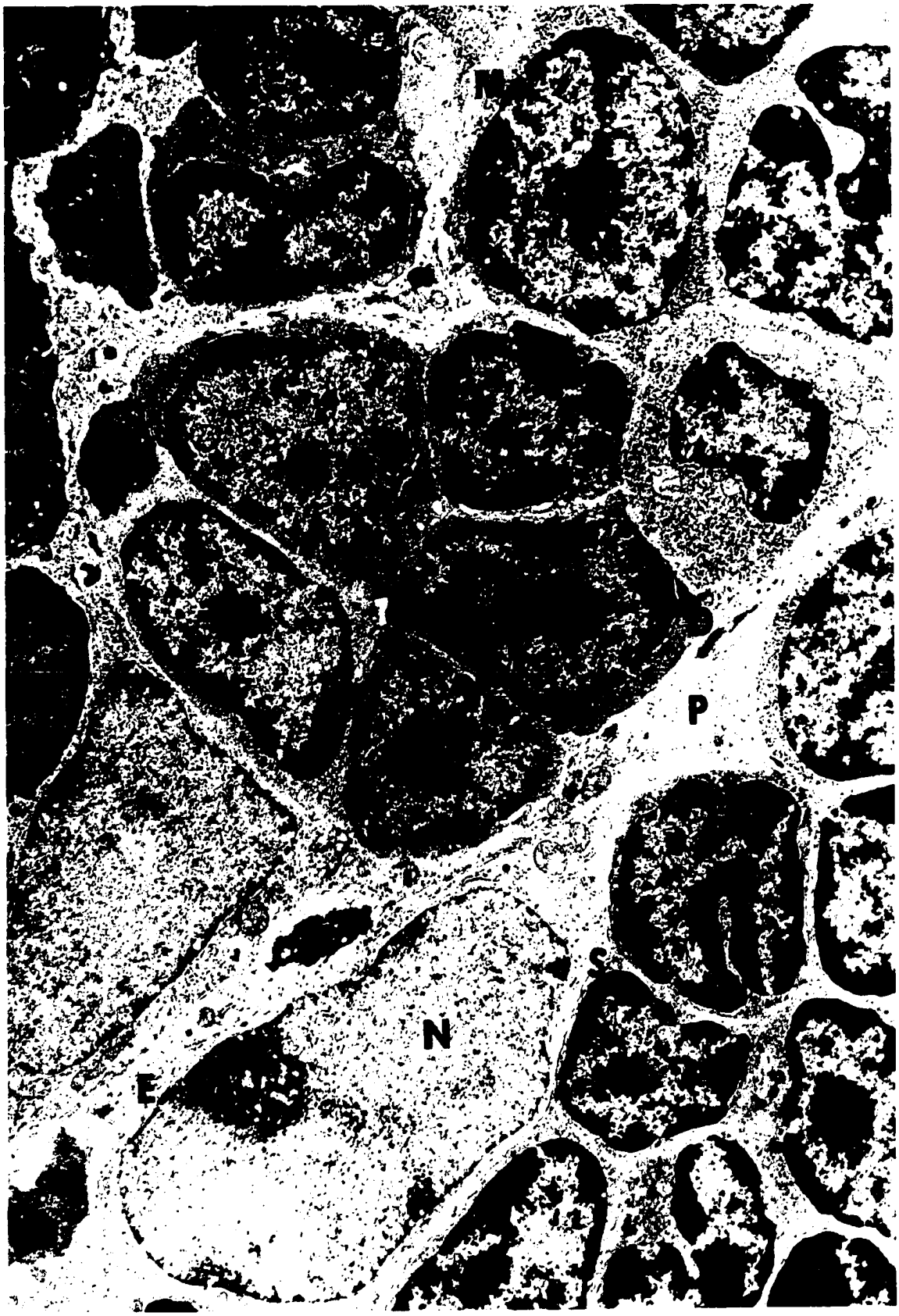

N
P
P
N
E

Figure 68. Control Animal, Study IV: Thymic cortex of control guinea pig sacrificed at 21 day interval post DNCB sensitization. The thymic cortex contains a large number of small cortical lymphocytes (S). The lymphocytes are clustered between the long thin epithelial cell (E) processes (P). These processes contain a few intracytoplasmic organelles, e.g., mitochondria (M), and electron dense bodies (ED). (uranyl acetate and lead citrate 5,750X).

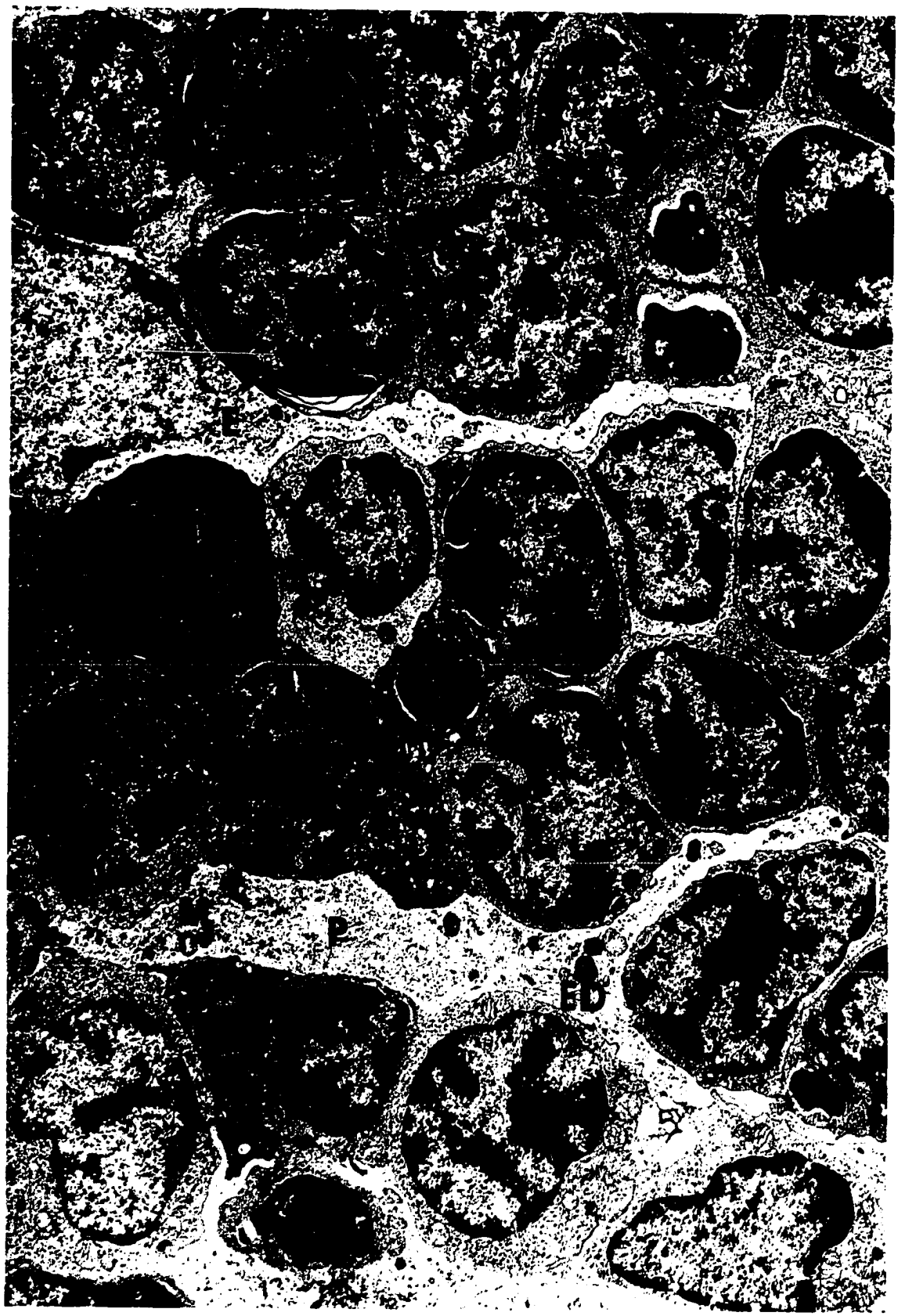

L
P
BD

Figure 69. Experimental Animal, Study IV: Thymic cortex of a procarbazine hydrochloride treated guinea pig sacrificed at 0 day interval post DNCB sensitization. The cortical region shows a mixture of small (S), medium (M), and large (L) sized lymphocytes. The nucleus and cytoplasm of these cells are morphologically unaltered. The epithelial cell cytoplasmic processes (P) are expanded and exhibit signs of morphological damage. (uranyl acetate and lead citrate 7,200X).

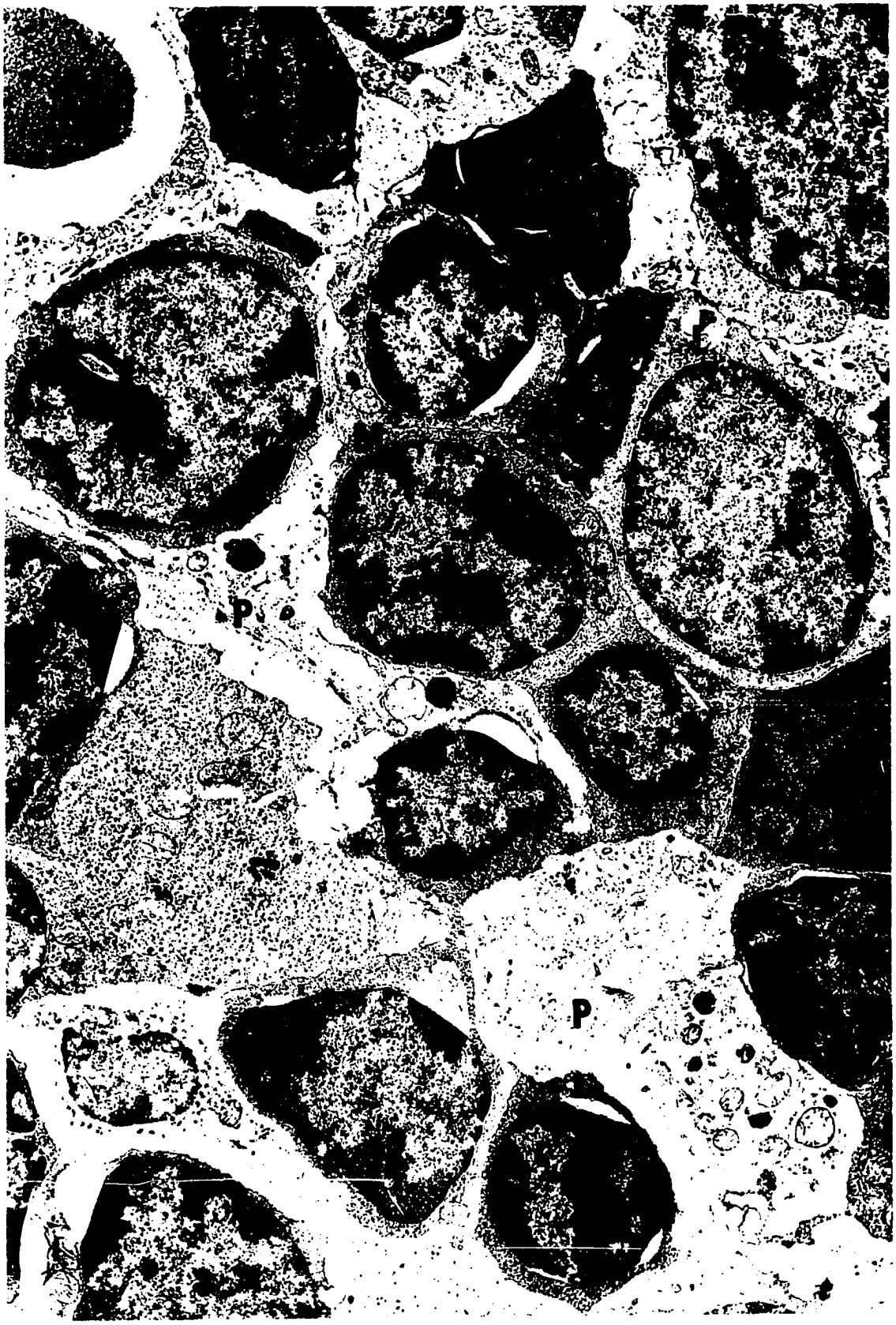
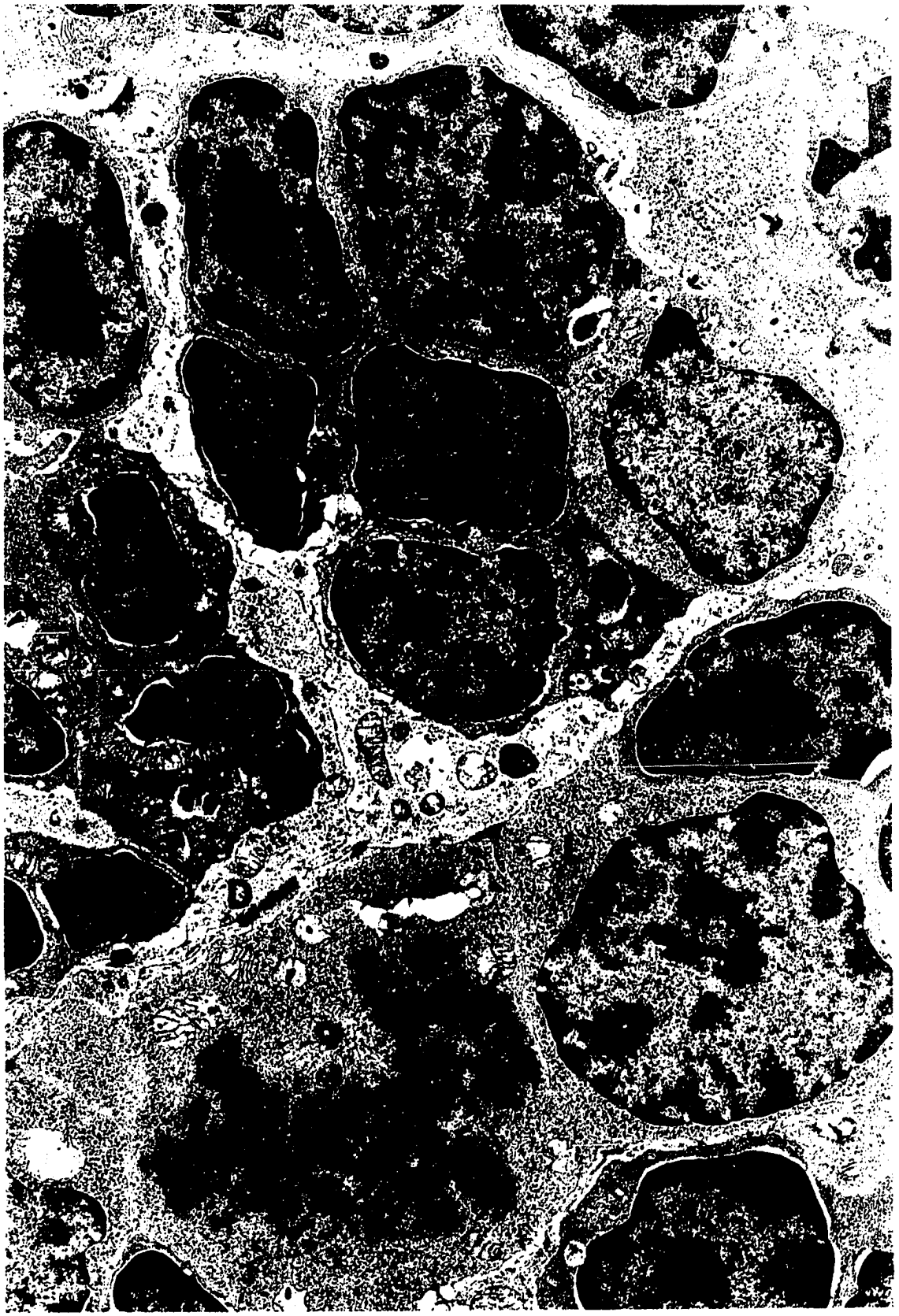

P
P

Figure 70. Experimental Animal, Study IV: Thymic cortex of procarbazine hydrochloride treated guinea pig sacrificed at 21 day interval post DNCB sensitization. The micrograph shows a mixture of small (S), medium (M), and large (L) sized cortical lymphocytes. The recovering thymic cortex displays an impressive number of large and medium sized lymphocytes. The epithelial cell cytoplasmic processes are comparable in appearance to those of control animals, i.e., thin, non-distended, few intracytoplasmic structures. Typical desmosomes (D) connect adjacent epithelial cell processes. (uranyl acetate and lead citrate 7,200X).



CHAPTER IV

DISCUSSION

Procarbazine hydrochloride (PH), a methylhydrazine derivative, has been reported to induce varying degrees of toxicity in different animal species (15, 30, 57, 74, 77, 85). This toxicity may present as alopecia, anemia, weight loss, respiratory distress or lethargy. In cases of exceeded maximum tolerated dosage or prolonged treatment with a tolerated dosage, the toxicity may induce animal death (8, 57).

Maximum Tolerated Dose Level

Experimentally, a maximum tolerated dosage of PH was set at a level which induced significant immunosuppression without inducing a lethality exceeding 20 to 25%. Maximum tolerated doses of PH appear to differ for each animal species (57, 85), but a pattern is presented in which maximum tolerated dosage levels for different species tend to decrease in quantity as the whole body weight of the animal increases. Stewart and Bell (85) reported a comparative study demonstrating maximum tolerated PH doses for mice, rats, and rabbits to be 120, 35, and 7.5 mg/Kg respectively. MacDonald, et al., (57) in a discussion of Scharer's (82) unpublished work, noted that dogs treated with 10 or 15 mg/Kg doses of PH experienced severe toxic effects and that prolonged treatment with a dosage of 5 mg/Kg of PH would induce severe toxicity. These investi-

gations established the importance of toxicity due to dosage and/or duration of PH treatment for different animal species.

Since the tolerated dosage level of PH treatment for guinea pigs was unknown, Study I was designed to obtain this information. Utilizing data obtained by previous investigators (57, 85) on mice, rats, rabbits, and dogs, Study I incorporated PH dosage levels of 15, 35, and 55 mg/Kg. Following chronic treatment (21 days) with these three doses, it was noted that guinea pigs, like dogs (57), had a narrow effective dose range. Guinea pigs treated with the two larger doses of PH either died during pregraft anesthesization (55 mg/Kg), or experienced unacceptable levels of toxicity (35 mg/Kg) while those animals receiving the lowest dosage of PH (15 mg/Kg) were free from signs of toxicity. The absence of toxic signs suggested that 15 mg/Kg was below the effective immunosuppressive level.

Mice and nonhuman primates treated with PH have been reported to exhibit severe degrees of physiological stress or death upon anesthesization with pentobarbitol (42, 76). This sensitivity was demonstrated during anesthesization for skin grafting in guinea pigs receiving chronic PH treatment with dosage levels of 35 and 55 mg/Kg. Since skin homografts were performed to evaluate the degree of immunosuppression in PH treated guinea pigs, the pentobarbitol sensitivity required an alternative method for evaluating cell-mediated immune suppression.

Immunosuppressive Dose Levels

It has been reported that the cellular infiltrate of first-set homograft rejections (83, 91, 92, 93) is comparable to that of DNCB sensitized skin test sites (28, 91). Skin sensitization with DNCB is a

method utilized for evaluating degrees of cell-mediated immunity in cancer patients undergoing chemotherapeutic treatment (14, 46). Considering the effects of anesthesization of PH treated animals, the similarity between cellular infiltrates of homografts and DNCB skin test reactivity, and the relevance of DNCB as a means of evaluating cell-mediated immunity in cancer patients, skin testing using the immunogen DNCB was substituted for skin grafting as a means for evaluating the degree of immunosuppression.

In Study II, guinea pigs were treated with an intermediate dosage of 25 mg/Kg to determine if this quantity of PH would induce immunosuppression to DNCB sensitization without exceeding a lethality of 20%. Following prolonged treatment (21 daily, plus 6 supplemental injections) these animals displayed marked immunosuppression to DNCB skin sensitization with minimal toxic effects. The minimal degrees of toxicity noted in these animals presented as retarded weight gain and mild signs of anemia. Although a pre-set maximum tolerated dosage with a lethality of 20% was not experienced in Study II, the available evidence, indicating a narrow dose range in other species (57, 85) and the data from Study I, indicated that the dosage level for guinea pigs should not exceed 25 mg/Kg.

Toxic Effects

Hematopoietic Changes

High dosage levels or prolonged exposure to PH treatment may induce toxic anemia. Methylhydrazine derivatives have been reported to induce anemia by hemolysis of erythrocytes (30). MacDonald, et al., (57) reported that prolonged treatment with a methylhydrazine derivative

(procarbazine hydrochloride) induced anemia in dogs by bone marrow depression. Floersheim and Brune (30) reported a 20% decrease in erythrocyte count in mice treated for prolonged periods with 120 mg/Kg of PH, and a more severe decrease when treated with 4 daily injections at a dose level of 360 mg/Kg. Quagliata, et al., (77) reported a less severe decrease (10%) in erythrocyte count in rats treated for 20 days at PH dosage levels of 30 mg/Kg. Possanza and Stewart (74) reported physical signs of anemia in rats treated with 35 mg/Kg/day for 21 days followed by 17.5 mg/Kg/day for 13 days. Physical signs of anemia (paleness of ears and eyes) were also demonstrated in guinea pigs receiving 21 daily injections of PH at dosage levels of 35 and 55 mg/Kg. Few experimental animals injected with the intermediate dosage of PH (25 mg/Kg) developed toxic anemia, and guinea pigs receiving 15 mg/Kg of PH did not demonstrate evidence of anemia. Animals developing anemia in the early stages of treatment almost invariably died prior to termination of treatment; however, if toxic anemia developed during the latter stages of treatment, the animals usually recovered after completion of treatment. Although direct blood studies were not performed to quantitate hematological alterations, indirect evidence indicated increased destruction of erythrocytes (increased hemosiderin in lungs and spleen) and increased extramedullary hematopoiesis (spleen).

Weight Changes

Procarbazine hydrochloride toxicity has been reported to induce animal weight loss (30, 77). The degree and rate of weight loss depends upon the animal species and dosage of PH given. Floersheim and Brune (30) reported a gradual weight loss (10%) in mice receiving 120 mg/Kg of PH

every other day for 23 days. However, they noted a precipitous weight loss (20%) in mice receiving 4 daily injections of 360 mg/Kg. Quagliata, et al., (77) reported initial weight loss of 19% in rats receiving 30 mg/Kg/day for 20 days. In both studies (30, 77), the treated animals regained weight upon termination of PH treatment.

Guinea pigs treated with large doses (35 and 55 mg/Kg) of PH experienced a severe weight loss which was similar to the effects of massive PH treatment in rats (30). Experimental animals receiving an intermediate (25 mg/Kg) or lower dosage (15 mg/Kg) of PH exhibited retarded weight gain as contrasted to severe weight loss induced by larger doses. The three exceptions in which experimental animals exhibited initial weight losses, noted after 7 days of PH treatment, followed thereafter by continuous weight gain may be attributed to early adjustments to drug toxicity (77) and/or to changes in their physical environment (repeated handling).

Respiratory Effects

Pulmonary alterations have been noted in patients receiving PH as a chemotherapeutic agent (21, 47, 55). Some of these alterations consist of pulmonary hypersensitivity, alveolitis, pleural effusion and pneumonitis. Physical signs of respiratory distress were noted in some guinea pigs receiving PH treatment but symptoms usually developed in animals receiving the larger doses (35 and 55 mg/Kg) or intermediate dosage (25 mg/Kg) of PH for prolonged periods of time.

Mortality

Studies involving mice treated with a maximum tolerated dosage of PH (30) gave an animal mortality of 22.8%. A 15% mortality was exper-

ienced in guinea pigs treated for prolonged periods of time with a dosage of 25 mg/Kg of PH. Of this 15%, the majority of animal deaths (66.6%) occurred in animals receiving the longest duration of treatment (21 daily, plus 6 supplemental injections).

Evaluation of Immunity

The skin sensitizing potential of DNCB in guinea pigs (23, 24, 28, 91) was substantiated in Study IV. This epidermally bound hapten (23, 24) was able to induce skin sensitivity to DNCB within 7 days after initiation of sensitization; the time interval required for sensitization was comparable to that reported by previous investigators (23, 24, 28, 90). The cell-mediated immunity demonstrated by skin testing at 24 and 48 hours (maximal development of erythema, induration, epidermal-dermal mononuclear cell infiltration) also correlated well with previous reports (28, 34, 91).

The effectiveness of PH treatment in suppressing the immune response before immunization has been demonstrated previously in experimental models of transplantation and adjuvant-induced autoimmune diseases in mice, rats, and rabbits (15, 74, 77, 85, 86). These findings have been reconfirmed in the present study and extended to include another experimental animal. Guinea pigs receiving 21 daily treatments of PH prior to DNCB sensitization and supplemental PH treatment during sensitization displayed marked degrees of cell-mediated immunosuppression. Depending upon the method used to evaluate the skin tests, the maximum degrees of suppression presented at various time intervals after completion of DNCB sensitization. The maximum immunosuppression was demonstrated at 0 time interval when evaluated by test site diameter, at 7 days post-

sensitization when evaluated by cellular infiltration and at 14 days post-sensitization when evaluated by degree of induration. The degrees of immunosuppression decreased with discontinuation of PH treatment except when using induration as the means of skin test evaluation.

Procarbazine hydrochloride has been reported to suppress or completely abolish thymus-dependent but not thymus-independent immunity in rats (57, 77) and mice (29, 86). The severe lymphopenia exhibited in these models (30, 57, 77) has been attributed to the lymphotoxic effects of PH. Quagliata, et al., (77) proposed that PH treatment would induce destruction of the thymus and thymus-dependent areas of peripheral lymphoid organs and demonstrated, light microscopically, cortical thymic lymphocyte depletion in rats treated with 20 daily injections of 30 mg/Kg of PH. Floersheim, et al., (32) demonstrated a severe depletion of cortical and medullary thymic lymphocytes in adult mice following 14 injections of a methylhydrazine derivative, Ro-4-6824 (100 mg/Kg), given every other day over a 28 day period. These data prompted Study II which was designed to examine sequential histological changes in the thymus of PH treated guinea pigs. The morphological changes were followed by light and electron microscopy.

Study III - Thymic Morphology During PH Treatment

Control Animals: Light and Electron Microscopy

Light and electron microscopic characteristics of the thymus glands from untreated guinea pigs of Study III were similar in morphology to those reported previously for mice, rats, and guinea pigs (1, 45, 49, 59, 60, 79).

Experimental Animals: Light Microscopy

Light microscopic examination of thymic tissue from guinea pigs treated with 7, 14, or 21 daily injections of PH substantiated the earlier reports on rats (77) and mice (32) by inducing a marked depletion of small, medium and large sized cortical lymphocytes. Although Quagliata, et al., (77) did not make specific reference to medullary lymphocyte depletion in the rat, Floersheim, et al., (32) detected medullary depletion in mice.

In the present study, guinea pigs receiving 7 and 14 daily injections of PH did not demonstrate marked medullary lymphocyte depletion. However, animals receiving 21 daily injections of PH did demonstrate a slight degree of medullary lymphocytic depletion. The differences between the thymic medullary changes in this study and in those of Floersheim, et al., (32) could be attributed either to a chemical difference in the methylhydrazine derivative utilized or to biological differences in the experimental animals.

Experimental Animals: Electron Microscopy

Cortical lymphocytes. Cowan and Sorenson (17), Malacarne and Castaldi (58), Abe and Ito (1), Hauk, et al., (41), and Gad and Clark (33) reported rapid severe depletion of cortical lymphocytes in adult mice and rats treated with the immunosuppressive drugs, hydrocortisone and cyclophosphamide, and with the stress-producing agent, endotoxin. Ultrastructural examination of the hydrocortisone and endotoxin treated mice indicated that the first significant evidence of damage occurred in the lymphocyte nuclei. The nuclear changes involved progressive chromatin condensation with the formation of pycnotic nuclei followed by death

(17, 33). Although the ultimate fate of the damaged lymphocytes was lysis, the early cytoplasmic alterations were reported to be minimal in the hydrocortisone treated mice (17). Cowan and Sorenson (17) stated that the depletion elicited by hydrocortisone treatment involved primarily the small cortical thymic lymphocytes and that maximal thymic involution occurred 2 days after the second of two consecutive injections of hydrocortisone given at 24 hour intervals, i.e., 3 days. At the time of maximal hydrocortisone response (3 days), the involuting thymic cortex contained only a few small thymic lymphocytes.

Electron microscopic examination of thymic tissue from guinea pigs treated with 7, 14, or 21 daily injections of PH also demonstrated severe cortical lymphocyte depletion but the PH-induced depletion involved equally the small, medium and large sized lymphocytes. Nuclear changes comparable to those described for hydrocortisone treated mice were not seen in the thymus of PH treated guinea pigs at any time interval examined. The cytoplasm of the residual cortical lymphocytes demonstrated only moderate evidence of cell injury even after 21 daily PH treatments. Occasionally, tightly interlocked cytoplasmic processes were noted between residual lymphocytes in PH treated guinea pigs, but the direct cytoplasmic communications described by Balboni (3) during cortisone-induced thymic involution in rats could not be demonstrated.

The demonstration of severe cortical lymphocyte depletion in PH treated guinea pigs with minimal evidence of intrathymic lymphocyte destruction was an unexpected finding. This apparent discrepency could possibly be explained by the length of time between the initiation of PH treatment and the first tissue collection (7 days) but this would require

almost immediate destruction and precipitous removal of all traces of cellular debris. Furthermore, phagocytic cells, polymorphonuclear leucocytes and macrophages, did not appear until the 14 day sample when the epithelial components exhibited the most severe damage. The actual cause for this discrepency can not be given at this time, but indirect evidence suggests a mass exodus of almost all cortical lymphocytes rather than destruction in situ. The persistence of a small residual lymphocyte population in the cortex of PH treated guinea pigs may be attributed to the proposed heterogeneity of the cortical lymphocyte population. Abe and Ito (1) proposed that approximately 5% of the cortical lymphocytes are medullary in nature both morphologically and functionally (long-lived). It may be significant that these residual lymphocytes did not begin to exhibit significant morphological changes until approximately 14 days of treatment when the cortical epithelial cell damage was most severe.

Medullary lymphocytes. Abe and Ito (1) reported in their ultrastructural study of hydrocortisone treated mice that the small medullary lymphocytes of experimental animals were comparable to those of untreated controls. Electron microscopic examination of medullary lymphocytes in guinea pigs treated with 7 daily injections of PH were unremarkable when compared to controls, but animals receiving 14 daily injections of PH did demonstrate minor degrees of medullary lymphocyte alteration.

The absence of early medullary lymphocyte alterations could be attributed to the postulated heterogeneity of the thymic lymphocyte population with a corresponding difference in drug sensitivity. Abe and Ito (1) have proposed that short-lived thymic lymphocytes have their residency within the cortex, whereas the long-lived cells may reside within the

medullary region. The observation that prolonged PH treatment is necessary to produce alterations in the population of medullary lymphocytes of guinea pigs may lend some support to their hypothesis since the medullary and residual cortical lymphocyte populations begin to show significant changes at the same time. It is tempting to speculate that the residual cortical lymphocyte population is medullary in nature and is indeed long-lived.

Cortical epithelial cells. Cowan and Sorenson (17) were unable to detect significant ultrastructural changes in the cortical epithelial cells of mice 24 hours after hydrocortisone injection. After a longer time period (3 days post-injection), they were able to demonstrate distinct ultrastructural alterations in the cortical epithelial cells. These alterations consisted of many small intracytoplasmic vesicles or of fewer large cystic vesicles. The cortical epithelial cell alterations noted in guinea pigs treated with 7, 14, or 21 daily injections of PH consisted of cytoplasmic hypertrophy, increased numbers of electron dense bodies, membrane bound bodies, cystic vesicles with or without intracystic granular material, and degeneration. It was noted that the most severely damaged cortical epithelial cells were found in animals treated with 14 daily injections of PH. It may be significant that the time of maximal epithelial cell damage coincided with the previously discussed changes in the residual cortical lymphocyte population and with the appearance of phagocytic cells.

Medullary epithelial cells. Although guinea pigs treated with 7 daily injections of PH did not demonstrate ultrastructural alteration of the medullary epithelial cells, animals injected with 14 daily injec-

tions of PH did demonstrate medullary epithelial cell alterations. These alterations were similar to the cortical epithelial cell degeneration noted in animals receiving 14 daily injections.

Cortical invasive cells. Castaldi, et al., (13) and Harris and Kugler (40) demonstrated the migration of granular leucocytes into the thymic cortex of mice and guinea pigs following treatment with cyclophosphamides or with rabbit anti-guinea pig thymus serum. Both groups (13, 40) felt that the purpose of the invading granulocytes was to phagocytize cellular debris. Cowan and Sorenson (17) reported the presence of leucocytes 4 days following the second injection in their hydrocortisone treated mice and described the invading leucocytes as being located in "--large thymic epithelial cell cysts".

Guinea pigs treated with 14 daily injections of PH also exhibited polymorphonuclear leucocytes within the cytoplasm of degenerating cortical epithelial cells. Despite their intracellular position and proximity to cortical epithelial cell debris, the leucocytes did not demonstrate marked morphological evidence of phagocytosis. The subcapsular macrophages which appeared in animals receiving 21 daily injections of PH did exhibit evidence of phagocytosis (lysosomal-like bodies). Although actual ingestion was not demonstrated for either the polymorphonuclear leucocytes or for the macrophages, it would be appropriate to suggest that their presence was stimulated by degenerating cells and that their function was to ingest such material.

Study IV - Thymic Morphology During Recovery from PH Treatment

Light and electron microscopic examination of thymus tissue from sensitized controls and sensitized PH treated guinea pigs revealed the

presence of small, medium and large sized cortical lymphocytes. The sensitized control animals demonstrated an increased proportion of small cortical lymphocytes when compared to the unsensitized control animals in Study III. The role of the thymus in cell-mediated immunity (63) and the evidence that DNCB stimulated a strong cell-mediated immune response in guinea pigs (28, 91) may have had an important influence on the small lymphocyte population noted in DNCB sensitized control animals. To demonstrate a more accurate immunogenic influence upon mature differentiation of thymic lymphocytes will require additional study.

By indirect observation, the greatest degree of thymic cortex cellularity correlated with the time of strongest skin test activity to DNCB sensitization. Sensitized control animals skin tested at 0 day interval post-sensitization and sacrificed 72 hours later demonstrated a more cellular thymic cortex than did sensitized control animals sacrificed 21 days post-sensitization. These control animals also demonstrated the strongest skin test response to DNCB sensitization as indicated by skin test diameters and degrees of mononuclear cell infiltration. The over all cellularity of the thymic cortex was greater in sensitized control animals sacrificed at 0 day interval post-sensitization than in sensitized control animals sacrificed 21 days post-sensitization. The actual proportion of small cortical thymic lymphocytes may have been larger in animals sacrificed at the later time interval. When evaluating thymic recovery in the PH treated animals, consideration must be given both to the role of antigenic stimulation and to the natural lymphocyte replenishment upon cessation of treatment.

Quagliata, et al., (77) demonstrated, light microscopically, that rats treated with 100 mg/Kg of a methylhydrazine derivative (Ro-4-6824) replenished the cortical and medullary regions of the thymus within 18 days after cessation of drug treatment. In mice, Cowan and Sorenson (17) reported an increase in the number of thymic epithelial cells and a reappearance of thymic lymphocytes (predominantly large lymphocytes) within 6 days following the last injection of hydrocortisone. Twelve days following the last injection of hydrocortisone, the mouse thymus was normal (17).

Light and electron microscopic examination of the thymus from guinea pigs receiving 21 daily injections of PH followed by DNCB sensitization and sacrifice at 0 day interval post-sensitization revealed replenishment of small, medium and large sized lymphocytes. The early lymphocytic replenishment of PH treated, DNCB sensitized animals in this study can not be compared directly to the work of Quagliata, et al., (77) or Cowan and Sorenson (17) because the animals were undergoing DNCB sensitization and were also receiving supplemental PH treatment during the 7 days in which lymphocyte replenishment occurred. Direct correlation of the rate of thymic replenishment in guinea pigs with the earlier studies of rats (77) and mice (17) would have required the use of PH treated animals without verified immunosuppression.

Central and Peripheral Lymphoid Interaction

The role of the thymus as a central lymphoid organ (63, 71) and its role in the development of peripheral lymphoid tissue has received a considerable amount of attention. Emigration of lymphoid cells from the thymus to peripheral lymphoid organs has been demonstrated in the

guinea pig (53, 54, 65, 67, 68). Linna (53) has reported that with DNCB stimulation of cell-mediated immunity in guinea pigs there is a change in thymus cell migration patterns to favor the lymph nodes within the areas of sensitization. He further emphasized that there is no increased proliferation of thymus lymphocytes, i.e., proliferation is confined primarily to the peripheral lymphoid tissues.

Unpublished observations on peripheral lymph nodes from DNCB sensitized guinea pigs in this study substantiate these observations of Linna (53). In DNCB sensitized guinea pigs, there was not a marked increase in the number of cortical thymic lymphocytes undergoing mitosis; however, the earliest thymic tissue removed from DNCB sensitized animals was 10 days following their first exposure to DNCB. This 10 day lag is probably too long to detect an increase in thymic lymphocytic mitoses. The overcompensation with small cortical lymphocytes in animals which were previously depleted of cortical lymphocytes prior to DNCB sensitization stimulates questions concerning the possible role of antigen stimulation on thymic lymphocyte proliferation.

Cowan and Sorenson (17) described an increase in thymic epithelial cells of mice 6 days after completion of hydrocortisone treatment. Electron microscopic examination of thymic tissue from PH treated guinea pigs sacrificed at 0 day interval post-sensitization demonstrated highly altered cortical thymic epithelial cells. These differences in epithelial cell morphology may be related to the fact that the animals in this study received supplemental PH injections to within 3 days of sacrifice.

CHAPTER V

SUMMARY AND CONCLUSIONS

The objectives of this study were: 1) to determine an effective immunosuppressive dose level of procarbazine hydrochloride (PH) for guinea pigs; 2) to demonstrate by light and electron microscopy the sequential changes in guinea pig thymus during PH treatment; 3) to compare the severity of thymic alterations with the degree of immunosuppression to dinitrochlorobenzene (DNCB) sensitization; 4) to correlate the recovery of a previously suppressed thymus with the recovery of the immune response upon cessation of PH treatment.

1. Guinea pigs treated with 21 daily injections of PH at dosage levels of 35 and 55 mg/Kg exhibited severe degrees of toxicity and high mortality; animals receiving 15 mg/Kg exhibited no toxic reactions and appeared to be unaffected. Animals receiving 25 mg/Kg of PH for 21 consecutive days followed by 6 supplemental injections every other day exhibited marked immunosuppression and manifested acceptable degrees of toxicity (mild anemia, retarded weight gain) and mortality (15%). The highest percentages of mortality occurred in animals undergoing the longest duration of treatment.

2. Guinea pigs treated with 25 mg/Kg for 7, 14, or 21 consecutive days exhibited marked cortical thymic lymphocyte depletion with

minimal evidence of *in situ* destruction. This depletion involved small, medium and large sized lymphocytes; depletion was less severe in the medulla. Cortical epithelial cells exhibited progressive morphological alterations with some degeneration *in situ* as early as 7 days after initiation of PH treatment. Cortical and medullary epithelial cells exhibited the most severe damage at 14 days.

3. Guinea pigs treated with PH for 21 consecutive days prior to DNCB sensitization demonstrated marked degrees of immunosuppression when skin tested at various time intervals post-sensitization. The time at which the greatest degree of immunosuppression occurred differed according to the method used to evaluate the skin test site, *i.e.*, diameter, induration, cellular infiltration.

4. Tissue from DNCB sensitized PH treated animals demonstrated replenishment of thymic cortex as early as 10 days following the last daily injection of PH and only 3 days after their last exposure to DNCB sensitization. This early replenishment involved a mixture of small, medium and large sized thymic lymphocytes. Thymi from PH treated animals sacrificed 28 days after completion of daily PH treatment and 24 hours after completion of DNCB sensitization demonstrated an overcompensatory replenishment of small lymphocytes.

These observations indicate that 25 mg/Kg of PH is an effective immunosuppressive dose level for guinea pigs and will induce severe cortical thymic lymphocyte depletion with epithelial cell damage. These thymic changes are accompanied by marked immunosuppression to DNCB sensitization. Replenishment of the thymic cortex with lymphocytes and recovery from the immunosuppressed state occur promptly upon withdrawal of PH treatment.

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