# Differences in the Peritoneal Response to Pristane in BALB/cAnPt and BALB/cJ Mice

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## INTRODUCTION

Plasmacytomas are readily induced in all sublines of Balb/c mice except Balb/c J following intraperitoneal injections of 2, 6, 10, 14 Tetramethylpentadecane (Pristane) (Potter et al, this volume). The molecular basis for resistance and susceptibility to plasmacytoma induction is not known but chromosomal aberrations (Ohno et al 1980 and 1984), viral ocogenes (Potter et al 1984) and growth promoting influences of granulomatous inflammation (Potter and MacCardle 1964) all appear to be associated with pathogenesis. Although the initial mutagenic event remains ellusive, generation of clastogenic oxidants and/or prostaglandin metabolites within oil-induced granulomatous inflammation may contribute to plasma cell tumor induction. This is further supported by the observation that the antiinflammatory drug indomethacin prevented development of plasmacytomas in susceptible strains (Potter et al, to be published). The present study examines cellular and microenvironmental features of oil-induced lesions in Balb/c sublines which differ with regard to susceptibility to Pristane-induced plasmacytomagenesis.

## MATERIALS AND METHODS

### MICE:

The Balb/c An Pt mice used in these studies were inbred offspring of mice originally obtained from Dr. Howard B. Andervont in 1964. Balb/c J Pt mice were bred from offspring of Balb/c mice purchased from the Jackson laboratory in 1977 and 1979. All mice designated Pt were housed in Dr. Potter's colony at Litton Bionetics, Rockville, MD. Additional Balb/c Jax mice were purchased from Jackson Laboratory and housed at USAMRIID, Frederick, MD

PLASMACYTOMA INDUCTION: Two month old mice were injected intraperitoneally (IP) with 0.5 ml or 1.0 ml Pristane (2, 6, 10, 14 Tetramethylpentadecane, Aldrich Chemical Co., Milwaukee, WI 53233). Balb/c An Pt mice were killed at sequential time points between the day of injection and 285 days to study development and evolution of peritoneal lesions.

MORPHOLOGICAL STUDIES OF MESENTERIC LESIONS: Groups of Balb/c An Pt and Balb/c J mice were killed at various time points after Pristane inoculation for gross examination of mesenteric lesions, routine histology, electron microscopy and examination of microvasculature following perfusion with Alcian Blue dye.

The entire mesentery from the distal duodenum through terminal ileum was removed intact and spread on absorbent paper in three

Current Topics in Microbiology and Immunology, Vol. 122

sectors. The mesenteries were fixed in 2% Glutaraldehyde in phosphate buffer pH 7.2, and examined under buffered saline using a dissecting microscope. All polypoid structures attached to each mesentery were counted and representative samples were removed for further processing prior to Scanning and Transmission electron microscopy (Anderson and Anderson 1976). The remaining tissues were processed for light microscopy.

For microvascular perfusion studies, mice were deeply anesthetized with pentabarbital, the thoracic cavity was opened and a solution of 1% Alcian Blue dye (Eastman Kodak) in normal saline was injected into the left ventricle after cutting the right atrium. Total body perfusion was indicated by blueing of the skin and the venous return at the right atrium. The mesenteries were removed and spread as above. The specimens were fixed, dehydrated in graded alcohols and cleared in Xylene prior to whole-mounting in Permount on 2"x2 1/2" glass slides.

## RESULTS

### Development of Mesenteric Lesions in Balb/c An Pt Mice

Intraperitoneal inoculation of Pristane induced a mild superficial fibrinopurulent exudate which covered the mesothelial surfaces of the mesentery, omentum and parietal peritoneum in the first 24 hours after inoculation. The exudate consisted of a fibrin matrix containing neutrophils, eosinophils, monocytes, oil-laden macrophages and lymphoid cells. Inflammatory cells and oil-droplet macrophages also accumulated in "so-called" milky spots which are non-encapsulated lymphatic tissues normally present within mesenteries (Holub M. et al).

The superficial exudate was organized into discrete patches separated by smooth areas of mesothelium by the fourth day after inoculation. The patches were more prominent along the adipose tissue surrounding major vascular bundles of mesentery and along the border between the mesentery and the small intestines. Active diapedesis of neutrophils, eosinophils, monocytes and lymphoblasts across open interendothelial junctions was evident in dilated postcapillary venules. Extravasated red cells were also present at these sites.

The distribution of cells within lesions at day 4 was largely random, except for palisaded clusters of neutrophils and eosinophils which surrounded free oil droplets (Fig. 1). We would like to call these structures polymorphonuclear (PMN) pulsars because of their radial structure and evanescent existance. Degranulating neutrophils and eosinophils juxtaposed to the oil droplet were structurally polarized and appeared to be depositing an electron dense precipitate at the oil/water interface. Macrophages replaced the neutrophils that were oriented around oil droplets except in larger oil droplets where the inflammatory cells were usually mixed. Macrophages containing oil droplets developed cytological characteristics of activation between day 4 and 8, e.g., prominant nucleolus, increased cytoplasmic volume, and increased density of polyribosomes and rough endoplasmic reticulum. Epithelioid cells and foreign body giant cells were not present until after day 8.

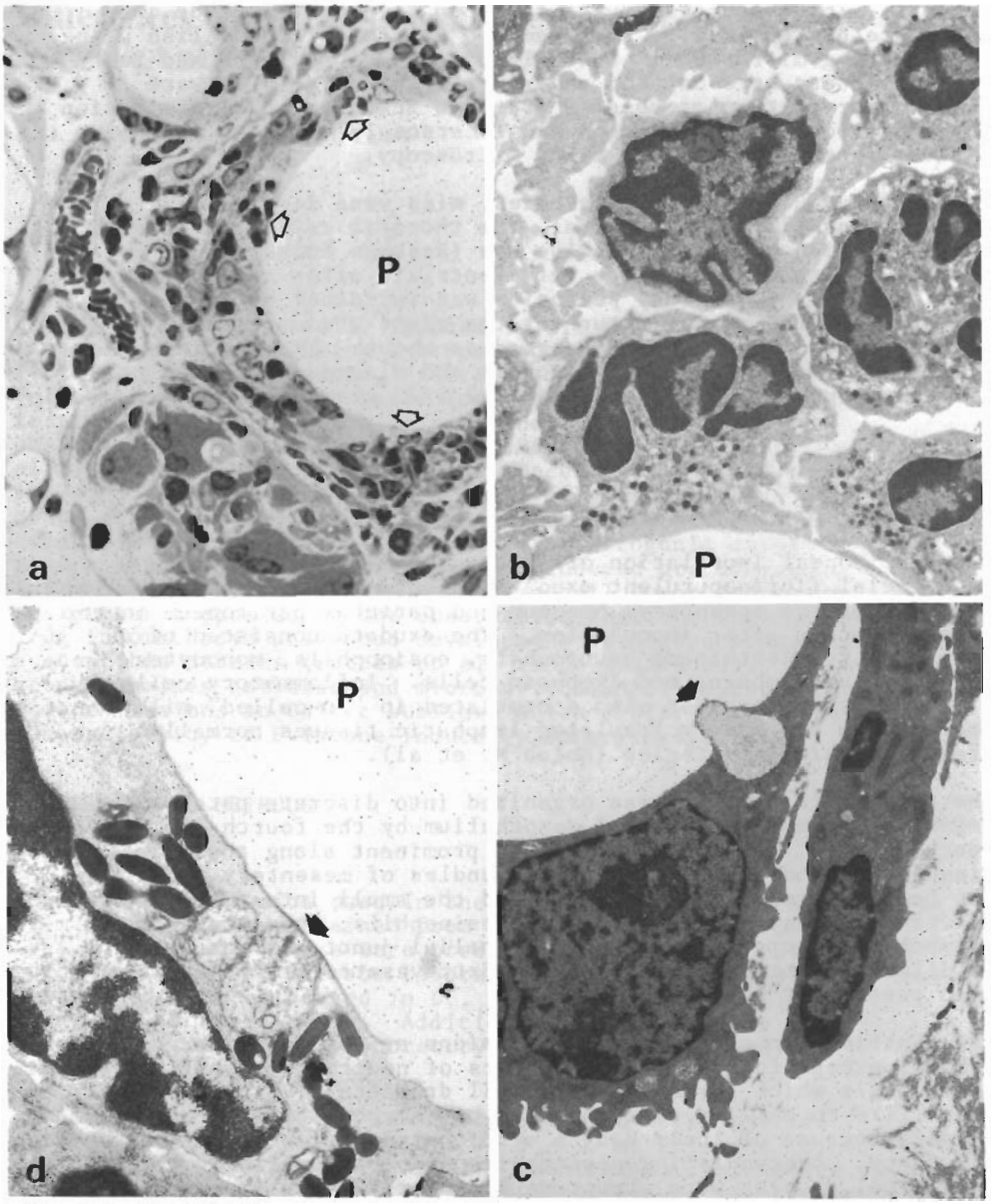


Fig. 1. (a-d) Polymorphonuclear (PMN) Pulsar (open arrows) surrounding a large oil droplet (p) in a Balb/c An Pt polyp contains mixed mixed inflammatory cells; the remaining pictures show neutrophils (b), a macrophage (c), and an eosinophil (d) depositing electron dense material (solid arrow) on a droplet of Pristane (P).

Table 1.
DEVELOPMENT OF MESENTERIC POLYPS IN BALB/c An Pt MICE

| DAY | #MICE | DOSE PRISTANE | #POLYP | OTHER LESIONS |
|---|---|---|---|---|
| 0 | 5 | 0 | 0 | Milky spots |
| 1 | 2 | 0.5mlx1 | 0 | Diffuse peritonitis |
| 4 | 2 | 0.5mlx1 | 0 | Organizing peritonitis |
| 8 | 2 | 0.5mlx1 | 0 | Oil granulomas |
| 12 | 2 | 0.5mlx1 | 2.0 | Polyps<granulomas |
| 15 | 3 | 0.5mlx1 | 3.6 | Polyps<granulomas |
| 45 | 3 | 0.5mlx1 | 18.0 | #Polyps significant |
| 67 | 3 | 0.5mlx1 | 82.0 | #Polyps significant |
| 93 | 7 | 0.5mlx1 | 71.0 | Early PCT focus in polyp |
| 150 | 2 | 0.5mlx2 | 219.8 | Diffuse PCT in peritoneum |
| 285 | 5 | 0.5mlx3 | 414.2 | High frequency of PCT |

Between eight and twelve days following Pristane inoculation the organizing mesenteric patches of exudate, containing a monotonous infiltrate of oil-distended macrophages, bulged from the surface of the mesentery like broad-based polyps. During this same time period the milky spots in the thin portion of the mesentery appeared to increase in number and size. Many of the milky spots and polyps contained epithelioid cells and foreign body giant cells after day eight which contributed to their resemblance to granulomas. Other milky spots exhibited foci of extramedullary myelopoiesis which had become more prominent following pristane inoculation.

The mesenteric oil granulomas located near vascular bundles became progressively more polypoid between 12 and 120 days; many were attached to the mesentery by delicate vascular peddicles which were best visualized in vascular perfusion studies or by scanning EM (Fig. 2). They were heterogeneous in size and stage of development since there was continuous induction of new lesions. The number of polyps increased from 2 to greater than 70 per mesentery by 93 days with the 0.5 ml dose of Pristane (Table 1). The frequency of polyps was markedly increased when multiple injections or higher doses of Pristane were used, however. At the later time points some of the polyps appeared to evolve from active cellular lesions containing viable oil-droplet macrophages and plasma cells to pearly-white acellular lesions containing collagen, calcium deposits, karyorhexis and ghosts of oil-laden macrophages.

Although there was heterogeneity with regard to development of polyps after day 30, acute inflammation in the form of PMN pulsars was present at all time points studied. The number of lymphoblasts and plasma cells in the interstitium between oil-laden macrophages and PMN pulsars slowly increased through emigration from the blood and local cell division, but clusters of small lymphocytes indicative of recirculation were rarely seen. Plasmacytoid lymphoblasts underwent mitosis in close proximity to high concentrations of degranulating neutrophils and "activated" macrophages (Fig. 3). Microscopic foci of large atypical plasma cells bearing intracisternal A-particles were

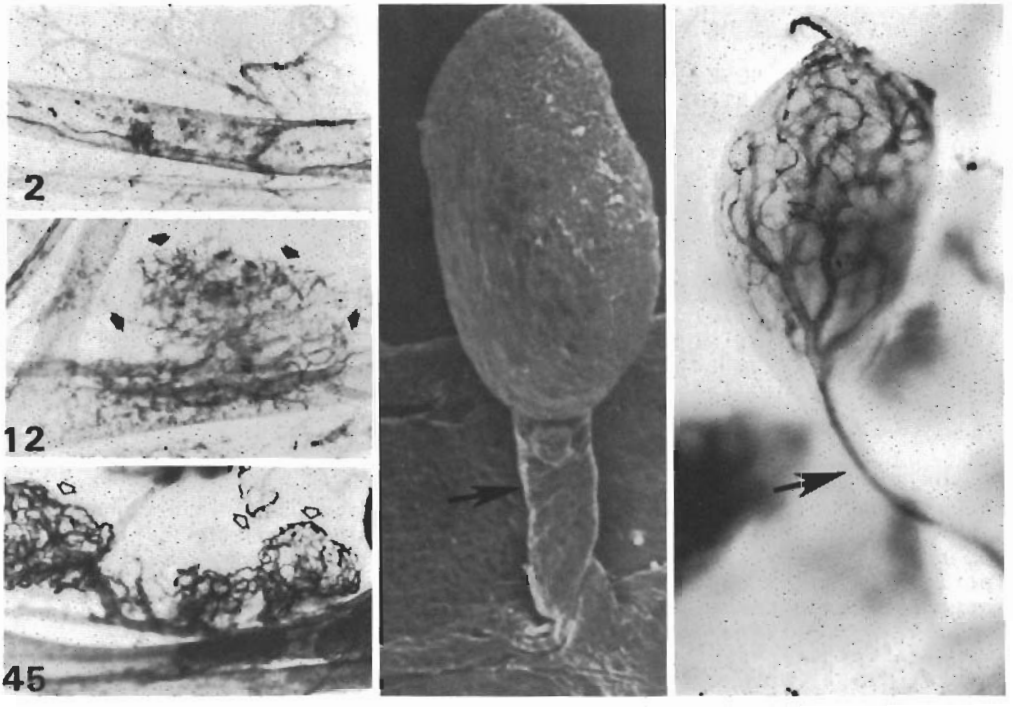


Fig. 2. The left hand column shows development of Pristane-induced angiogenic polyps studied by Alcian Blue dye perfusion 2, 12, and 45 days following intraperitoneal inoculation. The center and right hand columns show scanning electron microscopic and Alcian blue perfusion preparations of a fully developed mesenteric polyp. Note the vascular peddicle attaching the polyp to the mesentery (arrow).

found in some polyps as early as 93 days after Pristane inoculation, and locally spreading plasmacytomas were evident by 150 days.

Differences in Balb/c J mice

An extensive time course study was not conducted using Balb/c J mice, but, at 56 and 210 days following intraperitoneal injection of 1.0 ml Pristane there were nearly 10-fold fewer polypoid lesions on the mesenteries of Balb/c J mice compared to those of Balb/c An Pt (Table 2). The 5-31 polyps that formed in Balb/c J mice were all located at the border of the mesentery with the wall of the small intestine. In contrast, the 41-238 polyps of Balb/c An Pt mice were distributed over the central mesentery as well as the border.

Histogenesis of oil granulomas in Balb/c J mice were comparable to that of Balb/c An Pt mice with the important exception that

the polyps of Balb/c J mice contained tightly-packed clusters of small lymphocytes, non-phagocytic mononuclear cells with nuclear and cytoplasmic features of interdigitating dendritic cells (Steinman, RM, Nussenzweig, MC, 1980) and vascular structures resembling high endothelial venules (HEV) (Anderson et al 1976) normally found in peripheral lymphatic tissues (Fig. 4). The walls of the HEV-like venules were infiltrated with small lymphocytes in various stages of emigration and there were aggregates of small lymphocytes in the perivascular and interstitial spaces. At other sites in the Balb/c J polyps, structures resembling lymphatics were packed with cords of small lymphocytes. No structures analogous to Germinal follicles were found, however. In contrast, the cellular composition of Balb/c An Pt polyps appeared restricted to oil-droplet macrophages, epithelioid cells, plasma cells and acute inflammatory cells. Interdigitating dendritic cells, high endothelial venules, clusters of small lymphocytes in distended lymphatics and evidence of lymphocyte recirculation were virtually absent in Balb/c An Pt polyps.

Microvascular Alterations

Total body perfusion with Alcian Blue dye sharply delineated the microvasculature of the normal mesentery and the lesions that developed following Pristane inoculation. The normal vasculature in the mesenteries of Balb/c An Pt and Balb/c J mice did not differ in the absence of Pristane inoculation. Parallel vascular bundles consisting of an artery, vein and non-staining lymphatic plexus radiated from the root of the mesentery toward the wall of the small intestines where anastomotic arcades followed the contours of the intestine. These vascular bundles were covered by narrow strips of adipose tissue whose capillary networks were supplied by small arteries and veins that arose perpendicularly along the course of the larger vessels. Less frequent vascular sidebranches penetrated into the thin central area of the mesentery. Milky spots located in the central mesentery were invested with vascular networks organized in overlapping hexagonal arrays, including connections between narrow arterioles and dilated venous sinusoids. Milky spot vasculature was easily distinguished from Pristane-induced angiogenesis.

Terminal capillary loops in the periphery of the mesenteric adipose tissue of Balb/c An Pt mice were dilated and leaky 1-2 days after Pristane inoculation (Fig. 2). Between 2 and 4 days these sites became more opaque due to accumulation of exudate. By day 4 many vessels in these sites extended above the borders of the mesenteric adipose tissue and exhibited coiled or tortuous morphology indicative of angiogenesis. Between day 8 and day 12 the new vascular networks filled broad-based polyps. Within each polyp, the central supply vessels arborized into a peripheral submesothelial plexus of capillaries and small venules. The angiogenic vascular networks began to resemble glomeruli tethered to the mesenteric surface by long coiled peddicles by 45 days after Pristane inoculation (Fig. 2). At later time points the number and structural heterogeneity of the angiogenic mesenteric polyps increased markedly. Some polyps appeared avascular; other polyps retained a delicate basket-like superficial network of vessels; and, dense irregularly-dilated vascular networks were

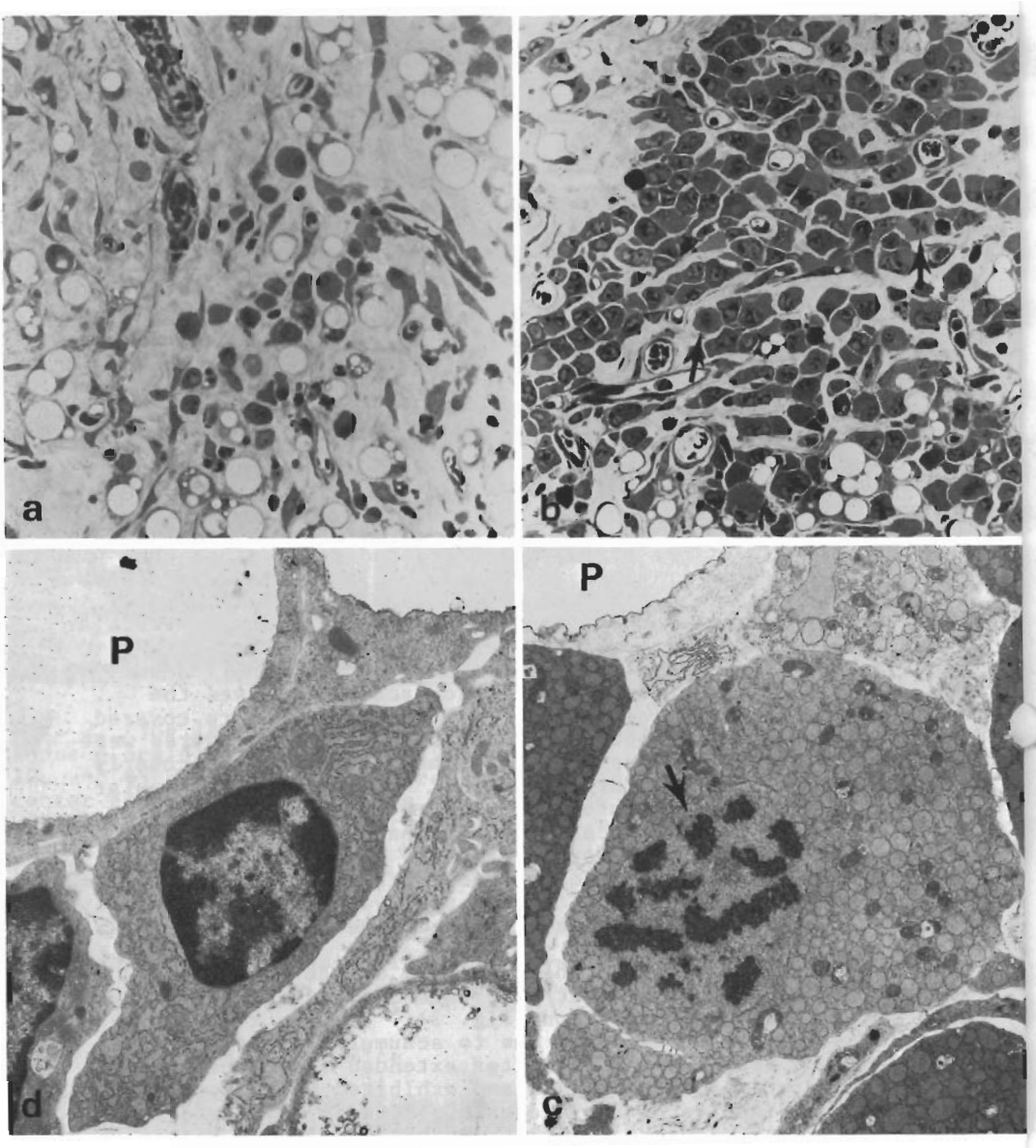


Fig. 3. In the Pristane-induced peritoneal lesions of Balb/c An Pt mice, plasmacytosis (a and d) results from a progressive increase in the number of normal plasma cells (arrows) located between oil-laden macrophages (p). Foci of atypical plasma cells (b and c), many with mitotic figures (arrows), may be found in Balb/c An Pt polyps as early as 90-150 days after Pristane inoculation.

associated with polyps or foci containing plasmacytoma cells. Indeed, the connective tissue matrix of well-established plasmacytomas consisted almost entirely of angiogenic vasculature and minimal perivascular connective tissue. Undoubtedly, such lack of support must contribute to the friability of plasmacytomas and the development of tumorous ascites.

Angiogenesis in Balb/c J mice was limited to the short 4 to 8 day interval following Pristane inoculation, after which time the new vessels regressed as the lesions organized. A few vascularized polyps developed near the intestinal border of the mesentery but virtually all the angiogenic activity was confined to local organization of the inflammatory exudate. The angiogenic response in polyps of Balb/c J mice included formation of venules with cobble stone luminal borders. There was at least one of these HEV-like vessels in 80% of the Balb/c J polyps compared to less than 1% for those of Balb/c An Pt mice.

Table 2.

DIFFERENCES IN MESENTERIC POLYP DEVELOPMENT
IN PLASMACYTOMA SUSCEPTIBLE AND RESISTANT BALB/c MICE

| STRAIN | #MICE | DOSE PRISTANE | DAYS | #POLYPS | PCT |
|---|---|---|---|---|---|
| Balb/c An Pt | 6 | 0 | 56 | 0 | (+) |
| Balb/c J Jax | 6 | 0 | 56 | 0 | (-) |
| Balb/c An Pt | 10 | 1.0 ml | 56 | 135.9 | (+) |
| Balb/c J Pt | 10 | 1.0 ml | 56 | 14.2 | (-) |
| Balb/c An Pt | 10 | 1.0 ml | 192 | 128.4 | (+) |
| Balb/c J Pt | 14 | 1.0 ml | 210 | 16.0 | (-) |

DISCUSSION

Intraperitoneal inoculation of Pristane induces a foreign-body granulomatous response in the peritoneal cavities of all mouse strains tested yet only Balb/c mice develop a high frequency of peritoneal plasmacytomas (Potter and Wax 1981, Potter et al this volume). The Balb/c J mouse which differs from the other Balb/c mice at only a few genetic loci, is resistant to plasmacytoma induction (Potter et al, this volume). The present study reveals additional features of the granulomatous response of Balb/c An Pt and Balb/c J mice that correlate with their respective susceptibility or resistance of plasmacytoma induction following Pristane inoculation. The results indicate that the normal progression of the inflammatory response to mineral oil, from acute inflammation through chronic inflammation to healing, is qualitatively altered in Balb/c mice.

Two elements of this process appear to predominate. In the Balb/c An Pt mouse the acute inflammatory component induced by Pristane persists too long and the healing phase is associated with exaggerated proliferative activity, manifested by angiogenesis and mesenteric polyp formation. Foci of acute inflammation (the PMN Pulsars) persist throughout the peritoneal

response in both sublines and contribute acute inflammatory mediators and reactive products to the local microenvironment. Evidence that angiogenesis, stromal cell proliferation, and extramedullary myelopoiesis are increased beyond what is needed for healing in the Balb/c Pt mouse, suggests strong local influence of growth factors which might be offset by inhibitory factors in the Balb/C J. Overextended growth of tissues involved in organization of the inflammatory site in the Balb/c An Pt mouse results in development of angiogenic polyps in which the earliest plasmacytomas develop.

The paucity of small lymphocytes in the polyps of Balb/c An Pt mice would not be obvious except when compared with the polyps of Balb/c J mice since both are chronic inflammatory tissue by definition, and both contain some small lymphocytes. The difference is qualitative and quantitative; there are more small lymphocytes in Balb/c J polyps and they seem to have arrived there by a process analogous to lymphocyte recirculation. The peritoneal lesions in Balb/c J mice contain dense aggregates of small lymphocytes in proximity with high endothelial venules (HEV) and interdigitating dendritic cells (IDC). Neither HEV nor IDC are present in Pristane-induced lesions in Balb/c An Pt mice. The significance of IDC and HEV is that they are structures normally found in the T-dependent cortex of lymph nodes (Anderson et al 1982). the IDC are believed to be the *in vivo* correlate of Ia-antigen bearing dendritic cells which are potent stimulators of T-lymphocytes *in vitro* (Steinman and Nussenzweig 1978). HEV are critically important to lymphocyte recirculation (Anderson and Anderson 1976). The specialized endothelial cells of HEV express undefined determinants on their luminal surfaces which are recognized by molecules on circulating lymphocytes causing the lymphocytes to adhere and emigrate (Chin et al 1982). Selective emigration of lymphocytes at HEV is fundamental to the continual repopulation of peripheral lymphatic tissues with lymphocytes which traffic throughout the body, and is the biological mechanism underlying clonal selection and antigen recognition *in vivo*.

Absence of trafficking small lymphocytes in the granulomatous polyps of the plasmacytoma susceptible mice, while the resistant subline displays abundant small lymphocytes, HEV and IDC, supports the proposition that small lymphocytes may somehow be involved in regulating the preneoplastic response of "transformed plasma cells" (Lynch etal 1979). The factors that are responsible for the deficiency of small lymphocytes in Pristane-induced polyps of Balb/c An Pt mice are unknown but differences in prostaglandin metabolism might be involved since indomethacin treatment converts Balb/c An Pt polyps to a lymphocyte rich phenotype (Anderson and Potter, unpublished) and prevents plasmacytoma induction (Potter et al, to be published). While the Balb/c An Pt mouse may be excessive in proliferative activity, the Balb/c J mouse has too much chronic inflammatory activity. Balb/c J mice produce too much alphafetoprotein (AFP) which is an acute phase reactant that would be elevated in the blood of patients with arthritis or other diseases of inflammatory nature.

Dr. Blankenhorn has found a defect in the regulator of AFP synthesis in Balb/c J mice which is normal in all other Balb/c

sublines (Blankenhorn et al, this volume) and Balb/c J mice develop a high frequency of arthritis following Pristane inoculation (Potter and Wax 1981) which is consistent with the hypothesis that this subline "over reacts" with regard to chronic inflammation. However, it is not known whether AFP levels are causally related to the arthritis or merely a sign.

Although angiogenesis is known to be stimulated by tumors (Folkman et al 1971), the angiogenic process in this model precedes tumor induction by hundreds of days. The extensive angiogenic response to granulomatous inflammation that develops on the mesenteries of Balb/c An Pt but not Balb/c J mice may contribute to the plasmacytomagenic environment. Increased vascularity of the peritoneal lesions in Balb/c An Pt mice may result in enhanced transport of inflammatory cells and oxygen leading to increased production of clastogenic prooxidants by inflammatory cells (Ceruttini 1985, Weitzman et al 1985). Dividing endothelial cells are known to release growth factors that specifically enhance division of plasma cells and hybridomas in vitro (Astaldi et al 1980). And, angiogenesis may mechanically contribute to the pedunculated shape of the lesions resulting loss of contiguity with the normal lymphatic microenvironments of the mesentery.

The resident population of cells in the polyps must be bathed in the metabolites and factors released by the predominant cell types, e.g., activated macrophages and polymorphonuclear leukocytes in various stages of degranulation. The clearance or detoxification of prostaglandins, interleukins, hematopoietic colony stimulating factors and mutagenic factors may be diminished by deposition of these factors on the droplets of Pristane This might cause local concentrations to increase over time. Deficiences in lymphatics could also result in accumulations of growth factors and mutagens. However, the fact that acute inflammation and oil granulomas were present in the mesenteries of either Balb/c sublines favors genetic differences in response to the factors rather than differences in production or clearance in plasmacytoma induction.

## SUMMARY AND CONCLUSIONS

The salient features of the Pristane-induced inflammatory response in Balb/c mice that correlate with plasmacytomagenesis are persistence of acute inflammation; angiogenesis and other tissue proliferative phenomena such as extramedullary myelopoiesis and mesothelial polyp formation; and, deficiency of immunosurveillance by small lymphocytes. In contrast, the Balb/c J subline differs from other Balb/c mice by exhibiting less proliferative activity and more infiltration with small lymphocytes.

Excess growth factors, mutagenic inflammatory products and deficient immune surveillance is a prime formula for tumor induction, but fortunately for the Balb/c mouse this environment seems to be present only in the angiogenic mesenteric polyps which develop following Pristane inoculation.

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