The Armed Forces Institute of Pathology
Department of Veterinary Pathology
WEDNESDAY SLIDE CONFERENCE
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CONFERENCE 8
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Conference Moderator: Dr. Victoria Hoffman, DVM, DACVP

CASE I – NOVARTIS CASE 2 (AFIP 3066019).

Signalment: Adult, male, CD-1 mouse (*Mus muscularis*)

History: An approximately 2-year-old, male, sentinel mouse was found dead with no premonitory clinical signs.

Gross Pathology: Gross findings included numerous white, firm masses up to 0.8 cm in diameter in the lung. Additional findings included numerous red masses up to 0.5 cm in diameter in all lobes of the liver and approximately 2 ml of blood in the abdominal cavity.

Histopathologic Description: The lung tumor was peripherally-located, had a glandular and papillary pattern, and was nonencapsulated and expansile resulting in compression of adjacent tissues. Glandular structures were lined with rows of cuboidal to columnar cells that enclosed central lumen and had only slight cellular atypia. Some areas had solid sheets of cells interspersed by cholesterol clefts. The tumor was multicentric (only observed in some submitted sections), with intrabronchiolar growth.

Areas of lung adjacent to the tumor had multifocal to coalescing inflammatory infiltrates composed of numerous large eosinophilic macrophages and multinucleate cells admixed with eosinophils, neutrophils, and lymphocytes within alveolar and bronchiolar spaces and associated with intra- and extracellular eosinophilic, acicular crystals.

Contributor's Morphologic Diagnosis: 1. Lung; Bronchioloalveolar adenocarcinoma, multicentric, mouse
2. Lung; Eosinophilic crystalline pneumonia, moderate, multifocal, mouse

Contributor's Comment: This animal had numerous spontaneous neoplasms including multicentric bronchioloalveolar carcinoma and hepatic hemangiosarcoma (the latter not submitted). Bleeding into the abdominal cavity from the hepatic tumors was the cause of death.

Eosinophilic crystalline pneumonia (ECP, formerly referred to as acidophilic macrophage pneumonia[3]) can occur spontaneously or in association with other pulmonary lesions such as infectious processes or tumors.[2] ECP can be subclinical to fatal and tends to increase in incidence with age and is more prevalent in specific strains of mice (highest incidence in 129S4/SvJae).[2]

The characteristic crystals in ECP were previously believed to be Charcot-Leyden crystals, a protein found in eosinophils and basophils.[3] Now it is known, however, that the crystals are composed of Ym1 protein, also referred to as T-lymphocyte-derived eosinophil chemotactic factor.[1] Ym1 is secreted by activated macrophages and is homologous to chitinase.[2] Its normal function is not well-defined, but is believed to be involved in host immune defense, eosinophil recruitment, and cell-cell and cell-matrix interactions consistent with tissue repair.[2] Macrophages activated by type 2 cytokines produce large amounts of Ym1.[2]

In addition to resulting in extravasation of eosinophils and recruitment of T cells, Ym1 crystals likely contribute to lung inflammation through mechanical damage and enzymatic degradation.[1]

AFIP Diagnosis: 1. Lung: Adenocarcinoma, CD-1 mouse (*Mus muscularis*), rodent.
2. Lung: Intraalveolar histiocytosis, multifocal, moderate, with abundant intracytoplasmic eosinophilic crystals (eosinophilic crystalline pneumonia).

Conference Comment: Eosinophilic crystalline pneumonia is a common background lesion of C57BL/6 background mice. In one report there was an 87% incidence with an overrepresentation of females in 129S4/SvJae mice.[2] It can be a spontaneous lesion, or associated with pulmonary adenomas, lymphoproliferative disease, allergic pulmonary disease and parasitic or fungal infections.[2]

There are four types of Ym proteins (Ym1, Ym2, Ym3, and Ym4). Although these proteins are members of the chitinase family of proteins, they do not possess any of the chitinase enzymatic activity.[2] Ym1 and Ym2 have approximately 95% of the same sequence identity but are expressed in different tissues. Ym1 is expressed in the lung and spleen, but not in the stomach, while Ym2 is expressed in the stomach, but not the lung or spleen.[2]

Lesions associated with eosinophilic crystalline pneumonia can range from subclinical to severe and fulminating. Three patterns of lung lesions predominate.[2] The first consists of diffuse interstitial inflammatory infiltrates of macrophages, multinucleate cells, eosinophils, lymphocytes, occasional neutrophils, with moderate to severe lymphoplasmactyic perivascular and peribronchiolar cuffing. The second consists of little to no crystals, with macrophage infiltrates localized to regions of a lung tumor. The third consists of focal to multifocal infiltrates localized around bronchioles with large rectangular crystals in the airways and minimal macrophage infiltrates.[2] The case presented in this conference appeared consistent with the second pattern of distribution and was unusual in that it had little to no interstitial reaction despite numerous intraalveolar macrophages with abundant eosinophilic crystals.

Pulmonary adenomas and adenocarcinomas are among the most common primary pulmonary neoplasms in mice. A-strain mice are particularly susceptible, and their development in this strain is usually associated with activation of K-*ras* within these tumors.[4] Other less common primary lung tumors within mice are squamous cell carcinoma, papilloma, neuroendocrine carcinomas, and adenosquamous carcinomas.[4]

The cell of origin for pulmonary adenomas and adenocarcinomas are thought to be either type II pneumocytes or Clara cells.[4] Ultrastructural features of Clara cell differentiation include apical cytoplasmic accumulation of smooth endoplasmic reticulum, which can be admixed with lamellar surfactant granule formation.

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CASE II – WIL-416051 (AFIP 3067175).

Signalment: Male, 2-year-old, Cynomolgus monkey (*Macaca fascicularis*)

History: This monkey was euthanized at the end of a 14 day toxicity study. There were no clinical observations.

Histopathologic Description: This is a section of kidney characterized by widespread, multifocal, peritubular infiltrates of predominantly lymphocytes and plasma cells. Adjacent tubules are often lined by epithelial cells with enlarged hyperchromatic nuclei or epithelial cells that are in various stages of degeneration and sloughing. Lymphoplasmacytic infiltrates are also present within or obliterate tubules and tubular basement membranes are breached. Rarely, large, eosinophilic, intranuclear inclusion bodies are present within tubular epithelial cells.

Contributor's Morphologic Diagnosis: Kidney: Interstitial nephritis, lymphoplasmacytic, multifocal, moderate with epithelial intranuclear inclusions.

Contributor's Comment: The histopathology and presence of intranuclear inclusions are consistent with a viral etiology, in particular polyoma virus. Several simian polyoma viruses have been described and compared to the JC and BK polyoma viruses in humans.[1] Polyoma viruses can cause latent infections in healthy hosts, but clinical disease occurs in immunocompromised hosts. Polyoma virus infection is a noted cause of severe nephritis and renal rejections in immunosuppressed renal transplant recipients. In clinically affected hosts, the virus can also cause progressive multifocal leukoencephalopathy. However, renal polyoma virus infections are often typically mild and self-limiting.

In this study, several monkeys in vehicle-treated groups as well as test-article treated groups had interstitial nephritis. Several female monkeys in this study also had mononuclear infiltrates (inflammation) of the smooth muscle of the gastrointestinal tract as evidence of infection, with no relationship to dose of test articles. Additionally, there were no clinical chemistry changes associated with renal disease or infection. Thus, we interpreted these findings to be incidental to the study.

AFIP Diagnosis: Kidney: Nephritis, interstitial, lymphoplasmacytic, multifocal to coalescing, moderate, with multifocal tubular epithelial karyomegaly and rare intranuclear inclusion bodies, Cynomolgus monkey (*Macaca fascicularis*), primate.

Conference Comment: Polyoma viruses and papilloma viruses are double stranded DNA viruses belonging to the Papovaviridae family of viruses. Several polyoma viruses, including SV40, simian agent 12, polyoma virus papionis-2 and lymphotropic papovavirus, infect old world primates. Closely related polyoma viruses in humans include BK polyoma virus and JC polyoma virus. All polyoma viruses share two regulatory proteins, known as the large T and small T antigen, which can serve as primers in PCR identification.[1] Clinically overt disease due to polyomaviruses are commonly associated with immunosuppression, often as a result of either infection with Simian Immunodeficiency Virus or the use of immunosuppressive drugs.

Cynomolgus Polyoma Virus, antigenically and genomically related to Simian Virus 40, has been reported to cause renal dysfunction and tubulointerstitial nephritis in immunosuppressed Cynomolgus monkeys. Intranuclear inclusions within karyomegalic (2-3X normal) tubular epithelial cells were a consistent finding within affected kidneys.[1]

The differential diagnosis for these inclusions includes cytomegalovirus with characteristic large, dense, intranuclear inclusions often surrounded by a halo (owl's eye cells), and adenovirus with very large intranuclear inclusions that are deeply basophilic, "smudgy", and not surrounded by a clear halo.

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CASE III – MK0610249 (AFIP 3069155).

Signalment: Young adult Rhesus macaque (*Macaca mulatta*)

History: The presenting animal was inoculated with simian influenza virus, SIVmac239, and simian human immunodeficiency virus, SHIVDH12 (MD14YE) on June 1, 2005. Eighteen weeks post inoculation, only SIVmac239 was detectable in the blood. Throughout the 64-week course of the study, CD4+ T cell levels consistently declined, ranging from 26% to <3% of cells/µL of blood. CD8+ T cell levels fluctuated, ranging as high as 73% of cells/µL of blood, then dropped severely between weeks 62 and 64 to 26% of cells/µL of blood. On August 22, 2006, week 63, the animal presented with a rapid respiration rate. Chest radiographs were obtained and results indicated diffuse pneumonia. Total

white blood cell count was 13,000 cells/µL of blood; lymphocyte count was 1248 cells/µL, monocyte count was 1040 cells/µL, and eosinophil count was 1287 cells/µL. The animal was euthanized.

Gross Pathology: Upon opening the chest cavity, the lungs did not collapse. The lungs were grayish-pink to light brown and were mild to moderately consolidated. Gross appearance of the lungs was consistent with diffuse pneumonia. The trachea contained a small amount of frothy white fluid. The spleen was mildly enlarged uniformly. No other abnormalities were noted in the other major organs or tissues.

Histopathologic Description: The lung exhibited diffuse alveolitis with alveoli containing foamy eosinophilic fluid with an admixture of small to moderate numbers of neutrophils, alveolar macrophages, multi-nucleated histiocytic giant cells, and numerous extracellular fungal organisms consistent with *Pneumocystis carinii*. Centrally to peripherally located within the fungal organisms was a small nucleus of the cystic and trophic forms of *Pneumocystis*. There was mild to moderate type II pneumocyte hyperplasia and within the septae, there was a mild mononuclear infiltrate comprised mainly of lymphocytes and a smaller number of plasma cells. Gomori methenamine silver (GMS) stains of the lung sections and imprint smears were positive for the cystic forms of *Pneumocystis carinii*.

Cytology: Lung imprints were prepared using a cut margin of lung stained with a modified Wright-Giemsa stain. Cytologic examination revealed red blood cells, alveolar macrophages, type II pneumocytes, neutrophils, and numerous round to oval extracellular organisms measuring approximately 4 µm in diameter. Nuclei were evident within the organisms and were consistent with the cystic and trophozoite forms of *Pneumocystis carinii*.

Contributor's Morphologic Diagnoses: Lung: Alveolitis, diffuse, acute with numerous fungal organisms consistent with *Pneumocystis carinii*.

Contributor's Comment: *Pneumocystis carinii* was once mis-identified as a protozoan, but was re-classified as a fungal organism based on mitochondrial genomic gene sequences, amino acid sequences of peptides and proteins, and comparative analysis of the 16 S ribosomal RNA.[5] Transmission of the fungus can be from host to host or from the environment.[15] Although *Pneumocystis* typically causes pneumonia in immunocompromised individuals, asymptomatic hosts may be carriers.[9,13,14,15] Immunocompetent hosts tend to develop transient *Pneumocystis* infections and can transmit infection to susceptible hosts by an airborne route.[6] *Pneumocystis* pneumonia is the most prevalent opportunistic infection in immunocompromised patients infected with human immunodeficiency virus (HIV) and is particularly difficult to study because the organism cannot be cultured outside the host lung; therefore, animal models continue to be the best source of information on *Pneumocystis*.[4,14] There are 5 host-specific species of *Pneumocystis*: *Pneumocystis carinii, Pneumocystis jiroveccii, Pneumocystis*

murina, Pneumocystis oryctolagi, and *Pneumocystis wakefieldiae*. *Pneumocystis carinii* species infects SIV positive macaques and the *Pneumocystis jiroveccii* species infects HIV positive humans.[1,7,12] Wild born and laboratory bred macaques have similar susceptibilities to *Pneumocystis carinii*, however, wild born macaques exhibit higher levels of antibody titers to the fungus.[8] The first report of infection in nonhuman primates included two aged owl monkeys and two young chimpanzees with myeloproliferative neoplasia.[3] Lungs affected with severe *Pneumocystis* pneumonia typically exhibit alveolar type-II cell hypertrophy and intra-alveolar foamy eosinophilic material and variable neutrophilic lung inflammation that may result in diffuse alveolar damage, impaired gas exchange, and respiratory failure.[6,14] The *Pneumocystis* fungi attach to type-I alveolar epithelial cells and, although the organisms do not alter the metabolic, barrier, or structural functions of the alveolar cells, they are able to proliferate due to impaired cell-mediated immunity. Infection also leads to changes in pulmonary surfactant production initiated by the inhibition of phosphatidylcholine from type II alveolar cells.[6] *Pneumocystis* organisms exist as cystic and haploid trophozoite forms throughout their life-cycle, but during infection, the trophozoite form is more abundant than the cystic form.[14] In the transmission electron micrographs of a thin section of *Pneumocystis* infected lung from this macaque, cyst and trophozoite forms are evident with trophozoites predominating. Free ribosomes and glycogen are present in both forms. Cysts are typically spherical in shape, contain up to eight intracystic bodies from which trophozoites originate, and have a mean diameter of 5-8 µm. The mean diameter of an intracystic body is 1-2 µm.[5] Each intracystic body contains components characteristic of eukaryotic cells including a centrally located nucleus, endoplasmic reticulum, and other organelles. The cyst wall is two layers thick, measuring approximately 50 nm.[5] Trophozoites are generally grouped into an interdigitating cluster, are individually pleomorphic, and are closely associated with pneumocytes. There are very few organelles, including a nucleus with a centrally to peripherally placed nucleolus. Free ribosomes predominate within the infective form. A 20-30 nm thick, non-homogenous coat surrounds the entire trophozoite, including the interdigitations, and is a characteristic feature of the trophozoite form. The coat may help anchor the trophozoite to other trophozoites or alveolar epithelium or it may be involved in nutrient uptake.[5] Trophozoites adhere to the alveolar epithelium to establish infection and to initiate a mitogen activated protein kinase signaling cascade for mating and proliferation of the fungus.[14] CD4+ T lymphocytes are integral in the defense against *Pneumocystis*.[6] By recruiting and activating macrophages and monocytes to attack the invading organism, CD4+ cells behave as memory cells to initiate the host's immune response.[14] Continuously declining CD4+ levels in this animal to <3% of cells/µL of blood allowed the *Pneumocystis* organisms to extensively proliferate and exert their adverse effects. SIV positive monkeys infected with *Pneumocystis* are unable to recruit CD4+ cells to the lung and develop an inflammatory response characterized by an increased neutrophil and CD8+ cell infiltration.[4] Not all of the imposed alveolar damage is due to the adherence of *Pneumocystis* organism to the type-I alveolar epithelial cells. TNF-α, an inflammatory cytokine that regulates the immune response by recruiting

neutrophils, lymphocytes, and monocytes to an area of infection, is also a contributing factor in damage to the alveoli. While TNF-α helps mount an immune response, it also releases oxidants, cationic proteins, and proteases responsible for subsequent damage to pulmonary tissue.[14] Trimethoprim-sulfamethoxazole combined with corticosteroid therapy to suppress lung inflammation is the preferred and most effective course of treatment for *Pneumocystis* and is usually begun when the CD4+ cell count is less than 200 per cubic millimeter, a state of a heightened risk of infection.[14] Adverse effects of the drug are common, however, and use of this prophylaxis has rapidly increased the multidrug resistance of infection bacterial pathogens found in human immunodeficiency virus-infected animals.[10,14]

AFIP Diagnosis: 1. Lung: Pneumonia, interstitial, histiocytic and neutrophilic, chronic, diffuse, moderate, with type II pneumocyte hyperplasia, multinucleate giant cells, and myriad intraalveolar fungi, etiology consistent with *Pneumocystis carinii*, Rhesus macaque (*Macaca mulatta*), primate.
2. Cytological specimen, impression smear, lung: Numerous epithelial cells, macrophages with vacuolated cytoplasm, few neutrophils, and myriad 3-5um round cysts containing punctuate organisms (trophic bodies) on a blue, granular, proteinaceous background.

Conference Comment: The contributor gives an excellent overview of *Pneumocystis carinii*. *Pneumocystis carinii* has also been described in pigs, foals, dogs, and other domestic animals with underlying immunosuppression.[2] The characteristic histologic finding is an eosinophilic foamy or flocculent material within alveoli with numerous intra- and extracellular fungal bodies. The organism can be more readily identified with Periodic acid-Schiff (PAS) procedure, or Gomori methenamine silver (GMS) stain.[2]

Contributing Institution: National Institutes of Health, Division of Veterinary Resources, Office of Research Services, Bethesda, MD

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CASE IV - CRL 1 (AFIP 3065936).

Signalment: 8-week-old, male, NZW rabbit, *Oryctolagus cuniculus*

History: Submitted for routine colony surveillance. No clinical signs reported

Histopathologic Description: Small intestine: More than 95% of epithelium is distorted by intracellular coccidia, including all villi and some crypts. Remaining crypts are compressed. The lamina propria has a slight infiltrate of lymphocytes, plasma cells, macrophages and heterophils. Parasitized mucosal cells have

marginalized nuclei and contain one of three phases. Some cells contain nonsporulated oocysts, also abundant in the lumen. Oocysts are approximately 30x15 to 30x20 microns, with a thick, dark, refractile, isotropic wall. A micropyle is visible on a few oocysts. Other epithelial cells contain macrogametocytes or microgametocytes in various stages of development. In general, the less developed forms are present deeper in the crypts. All coccidial forms appear limited to the lumen and the epithelium.

Contributor's Morphologic Diagnosis: Coccidiosis diffuse, severe, with severe loss of absorptive epithelium and mild, subacute enteritis.

Contributor's Comment: *Eimeria magna* was detected by fecal centrifugation and concentration on this rabbit. *Eimeria perforans* was detected in another rabbit from this cohort, so all likely had a mixed infection.

Despite the striking degree of mucosal involvement, no diarrhea was reported, and no evidence of it was noted at necropsy. Nonetheless, *E. magna* is reported to be of moderate pathogenicity.[4]

Intestinal coccidiosis continues to be common in rabbits, probably due to multiple factors, including the frequency of inapparent infections, the massive number of oocysts shed by infected rabbits, and the resistance of oocysts to many disinfectants. More than 12 species of intestinal coccidia have been reported in rabbits,[6] most of which live in the small intestine. Definitive diagnosis requires examination of sporulated oocysts. In our experience, oocysts will sporulate in fecal samples left at room temperature (presumably as in the wild) or in the refrigerator for several days, but sporulation is more reliably accomplished by incubation at room temperature for 1-5 days in a potassium dichromate solution. Sporulated oocysts are readily speciated based on size, appearance, and the appearance, if present, of the micropyle and residual body.

The life cycle of *E. magna* is typical of *Eimeria* spp. All *Eimeria* are host-specific and have a direct life cycle.[2] Oocysts are not infective until sporulation, so ingestion of cecotroph feces does not result in autoinfection. Ingestion of sporulated oocysts (sporocysts) results in release (excystation) of sporozoites. These invade enterocytes, round up and form trophozoites, and multiply asexually by schizogony (merogony), forming schizonts (meronts) that may contain more than 100,000 merozoites. Merozoites escape the host cell, resulting in death of that cell. Each merozoite can then invade another host cell for the next generation. The number of asexual generations is characteristic of each *Eimeria* sp., *E. magna* has been variously reported as having four or five[3,5] asexual generations prior to gametogony. In gametogony, the final generation merozoites form either macrogametocytes (female) or microgametocytes (male). Macrogametocytes have a single nucleus, and numerous peripheral PAS-positive granules. Microgametocytes are multinucleate. Each nucleus becomes incorporated into a small biflagellate sperm-like microgametocye. After

fertilization by the microgametocyte, the macrogametocyte develops into an oocyst. It has been estimated that one oocyst of *E. magna* can produce more than 25,000,000 oocysts in a susceptible host.[4] Given that feces from asymptomatic rabbits may contain more than 400,000 oocysts/gram, the potential for massive infection is apparent. Clinical disease is thought to result primarily from loss of functional mucosa and loss of mucosal barrier integrity as cells are lost.

We personally find it interesting that such massive infection did not cause significant debility (note the normal mesenteric fat) or diarrhea. The general sparing of the crypts may indicate that the rabbit retains sufficient epithelial replacement capacity, although no increase in mitotic rate was noted. Sparing of the crypts and the observation of generally less developed forms deeper along the villus also suggest two evolutionary adaptations of this parasite: first, that sparing the crypts is advantageous to the replication of the parasite as the host is not rapidly killed; and second, that invasion of cells near the crypt or along the side of the villus may be preferable as those cells are less likely to be shed prior to completion of a particular phase in the life cycle.

AFIP Diagnosis: Small intestine, mucosa: Coccidial macrogametes, microgamonts, and oocysts, intraepithelial and intraluminal, myriad, New Zealand white rabbit (*Oryctolagus cuniculus*), lagomorph.

Conference Comment: The Family Eimeriidae includes *Eimeria* and *Isospora*. Coccidia of domestic animals are relatively host and tissue specific. A table listing the common *Eimeria* and *Isospora* species of animals and the tissues in which they are found has been included below for quick reference.

***Eimeria* and *Isospora* of Animals**		
Geese & ducks	*E. truncata*	Kidney
Sandhill whooping cranes	*E. reichenowi*	Disseminated
Parrots	*E. psittaculae*	Intestine
Chicken	*E. acervulina*	Duodenum
Chicken	*E. necatrix*	Mid-intestine
Chicken	*E. tenella*	Ceca
Cattle	*E. bovis*	Small intestine, cecum, colon
Sheep	*E. ashata*	Small intestine
	E. bakuensis	Small intestine
	E. ovinoidalis	Ileum, large intestine
Goats	*E. Christenseni*	Small intestine
	E. arlongi	Small intestine
	E. ninakohlyakimovea	Large intestine

Horses	*E. leukarti*	Small intestine
Swine	*I. suis* *E. debliecki* *E. porci* *E. scabra*	Intestine
Dogs	*I. canis*	Ileum, cecum occasionally
Cats	*I. felis*	Small intestine, colon occasionally
Mice	*E. falciformis*	Colon
Rabbit	*E. stiedae* *E. intestinalis* *E. flavescens*	Bile ducts Ileum, cecum Ileum, cecum
Guinea pig	*E. caviae*	Large intestine
Ferret	*E. furonis*	Gallbladder, bile duct

Conference participants briefly reviewed the coccidian life cycle. Oocysts are shed in feces and sporulate. The oocysts of each species are morphologically distinct, but share similar features. The oocysts of *Eimeria* have four sporocysts, each with two sporozoites, with a total of eight sporozoites in each oocyst. The oocysts of *Isopora* have two sporocysts, each with four sporozoites, with a total of eight sporozoites in each oocyst. Ingested sporozoites excyst in the intestine and invade epithelial cells where they round up and form trophozoites. Asexual replication or schizogeny follows forming schizonts containing merozoites. The schizonts rupture, releasing the merozoites, which infect other epithelial cells and continue to replicate. Merozoites eventually form sexual stages (male-microgamete, female-macrogamete) which unite to form oocysts.[2]

Conference attendees also reviewed the ultrastructural features of apicomplexans, specifically *Toxoplasma*: parasitophorous vacuole, rhoptries, micronemes, apical conoid, apicoplasts, and dense granules.

This case was reviewed in consultation with Dr. Chris Gardiner, AFIP consultant in veterinary parasitology. We are grateful to Dr. Gardiner for his comments and advice on this interesting case.

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