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Role of Cell-Mediated Hypersensitivity in Budgerigar Breeder's Lung

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KONISHI, K., ARAI, S. and TAKISHIMA, T. *Role of Cell-Mediated Hypersensitivity in Budgerigar Breeder's Lung.* Tohoku J. exp. Med., 1981, **133** (2), 145–154 —— Eight subjects who possessed precipitating antibodies against budgerigar serum and budgerigar dropping extracts were investigated for the reactivity of their lymphocytes to the antigen. Lymphocytes from the five symptomatic breeders were significantly stimulated by budgerigar serum, but lymphocytes from the three asymptomatic breeders were not stimulated by this antigen, even though both groups showed precipitating antibodies. To investigate the participation of the T-cell response against budgerigar serum, the peripheral blood lymphocytes were fractionated into T-cells, B-cells and monocytes. Only the T-cells responded to the antigen when the monocytes were present. We concluded from these studies that the T-cell-mediated immune response together with a significant contribution from the monocytes plays an important role in the pathogenesis of bird breeder's lung. —— hypersensitivity pneumonitis; bird breeder's lung; T-lymphocytes; monocytes; lymphocyte proliferation

Hypersensitivity pneumonitis is generally considered to be caused by the repeated inhalation and subsequent sensitization to a wide variety of organic dusts such as avain proteins, fungi, thermophylic actinomycetes, animal and insect proteins (Fink et al. 1968; McCombs 1972), and perhaps also by some relatively simple molecular structures (Butcher et al. 1976; Fink and Schlueter 1978). Bird breeder's lung is one of those allergenic lung diseases induced by exposure to bird proteins derived from bird droppings and dander for certain periods of time (Reed et al. 1965; Hargreave et al. 1966). The clinical features of acute episodes of bird breeder's lung are characterized by fever, dyspnea, cough, rales, leukocytosis, and restrictive pulmonary function deficits within 4 to 6 hr after intensive exposure to avian antigens. Patients with recurrent exposure to bird droppings and dander often progress to a chronic form of the disease characterized by cough, fatigue, anorexia, weight loss, and pulmonary fibrosis. Lung biopsies have shown interstitial granulomatous lesion in the more acute cases, and interstitial fibrosis predominantly in the chronic cases.

There are many more immunological studies about bird breeder's lung than about other types of hypersensitivity pneumonitis. The immunopathogenesis of this disease is believed to implicate either Type III or Type IV hypersensitivity mechanisms, or both (Caldwell et al. 1973; Hansen and Penny 1974; Roberts and

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Moore 1977; Roska et al. 1979). Several recent studies, suggesting the direct role of sensitized T-cells, have demonstrated that the cell-mediated immune response might be closely related to the pathogenesis of bird breeder's lung (Schuyler et al. 1978; Sennekamp et al. 1978).

The present study was undertaken to demonstrate the direct role of sensitized T-cells and the significant role of monocytes in the activation of T-cells for the mediation of budgerigar breeder's lung.

Materials and Methods

Subjects. Fifteen subjects were studied: Eight subjects had precipitating antibodies against budgerigar serum and budgerigar dropping extracts; and the control group, seven subjects, had neither precipitating antibodies against these antigens nor any experience of breeding birds. Five of the first group were symptomatic and three were asymptomatic. The symptomatic and asymptomatic breeders were distinguished by clinical symptoms, chest x-ray films, and the inhalation challenge test with budgerigar serum. All of the patients in the first group revealed fine nodular infiltrates on their chest x-rays. Detailed information about these subjects is shown in Table 1.

Table 1. *Details of eight subjects*

Subjects	Age (years)	Sex	Bird exposure	Symptoms and duration
Symptomatic breeders				
Case 1	49	F*	Breeding budgerigars for 3 years	Cough, dyspnea, fever for a year
Case 2	51	F	In the bird shop business for 20 years	Dyspnea, fatigue, fever for 2 years
Case 3	30	F	Breeding budgerigars for 8 years	Cough, dyspnea, fever for a year
Case 4	38	F	Breeding budgerigars for 6 years	Cough, fever, sputum for a half year
Case 5	45	F	In the bird shop business for 7 years	Cough, dyspnea, fatigue for a half year
Asymptomatic breeders				
Case 6	56	M†	Husband of Case 2, and in the bird shop business for 20 years	No symptoms
Case 7	24	F	Daughter of Case 2, and has been living in the same house with her parents	No symptoms
Case 8	33	M	Breeding budgerigars for 3 years	No symptoms

* Female. † Male.

Inhalation challenge test. The inhalation challenge test was performed by the method described by Warren et al. (1977). Eight subjects inhaled 2 ml of a 1:10 dilution of budgerigar serum through a deVilbiss nebulizer. All of the symptomatic breeders developed typical symptoms — increasing dyspnea, dry cough, and fever accompanied by a restrictive defect in lung functioning — 4 to 6 hr after the inhalation of budgerigar serum. The asymptomatic breeders, however, did not develop any symptoms even when they inhaled increased doses of budgerigar serum up to 2 ml of a 1:5 dilution.

Immunological studies

Antigens. Budgerigar serum was obtained from 20 budgerigars by amputating the axillary artery. The serum was sterilized by millipore filtration and stored in aliquots at –20°C until it was used. After thawing, the serum was heat-inactivated at 56°C for 30 min prior to use. The budgerigar dropping extracts were prepared from freshly collected droppings according to the procedure outlined by Barboriack et al. (1965).

Immunodiffusion test. The immunodiffusion test was carried out by Ouchterlony's method in agar gel (Agarose Behringwerke AG) (Barboriack et al. 1965).

Cell fractionation and cultures. The mononuclear leukocytes were separated from the heparinized whole blood by centrifugation on a Ficoll-Isopaque gradient (Thorsby and Brattie 1965), and the interfacing mononuclear cells were used for peripheral blood lymphocytes (PBL). Differential cell counts from the PBL preparation gave values of 90–95% lymphocytes, 5–10% monocytes, and 0–1% neutrophils. The cell viability was 98% or more as assessed by trypan blue exclusion.

The monocytes were removed from the heparinized whole blood by allowing them to ingest carbonyl-iron and separating them on a Ficoll-Isopaque gradient (Lundgren et al. 1975). After the depletion of monocytes from the peripheral blood, the monocyte-depleted fraction contained less than 1% of positive peroxidase cells (Kaplow 1975). For the fractionation of the monocyte-depleted PBL into T- or B-cell subpopulations, the cells were resuspended in fetal calf serum (FCS) at 1×10^6/ml final concentration, with a 25:1 ratio of neuraminidase treated sheep erythrocytes (SRBC) per lymphocyte, and incubated at 0°C for 120 min in order to form spontaneous SRBC rosettes. The cell pellets were gently underlaid with Ficoll-Isopaque and centrifuged. The pellets and the interfacing cells were collected separately, and both were treated with 0.83% Tris-NH_4Cl to lyse the remaining SRBC. The cell fraction that remained at the interface after the centrifugation over the Ficoll-Isopaque gradient was designated as B, while the fraction that was pelleted was designated as T.

The peripheral blood monocytes were prepared by the following maneuver described by Kumagai et al. (1979): Briefly, 3 ml of heat-inactivated FCS were introduced into a plastic petri dish (No. 3002, Falcon Plastics) and allowed to stand overnight in a refrigerator. The dish was rinsed three times with phosphate buffered saline (PBS). Approximately 1×10^7 PBL in 4 or 5 ml of RPMI1640 medium with a 10% FCS was introduced into the serum-coated dish and incubated at 37°C in a 5% CO_2 incubator for 60 min. After incubation, the non-adherent cells were decanted and the dish was rinsed five times with cold RPMI1640 containing 10% FCS in order to eliminate completely the non-adherent cells. The adherent cells were collected from the dish by incubation at 4°C with PBS containing 0.2% EDTA and 5% FCS, and were washed three times with RPMI1640. The purity of the monocyte preparation was 96% as assessed by Baker's yeasts and Miller's method for assaying phagocytosis of yeasts (Miller 1969). The cell viability was 98% or more as assessed by trypan blue exclusion.

Cell cultures and DNA assays. Each group of fractionated cells was washed and their numbers were adjusted to a concentration of 5×10^5 cells/ml in a RPMI1640 medium with 10 % heat-inactivated FCS and antibiotics. They were subsequently incubated in 0.2 ml triplicated samples at various concentrations of budgerigar serum in microtest tissue culture plates (No. 3040, Falcon Plastics) at 37°C in a 5% CO_2 humidified environment for 7 days. 0.8 μCi of ^{3}H-thymidine, with a specific activity of 10 Ci/mmole (New England Nuclear), was added 4 hr before the cells were collected for analysis.

HLA typing. The HLA type was determined by using the standard microlymphocyte cytotoxicity technique of Terasaki and McClelland (1964).

Results

The immunodiffusion test. An analysis of sera from the symptomatic and asymptomatic breeders revealed the following: Sera from the symptomatic

breeders contained precipitating antibodies against budgerigar dropping extract in a ratio of serum to solution from 1:16 up to 1:256 in titers of two-fold dilution; and against budgerigar serum from 1:8 up to 1:64 in titers of the same dilution (Table 2). Sera from asymptomatic breeders contained precipitating antibodies against budgerigar dropping extract in a ratio of serum to solution of 1:128; and against budgerigar serum from 1:8 up to 1:64. Consequently, the presence of a high titer of antibodies to budgerigar antigens is not necessarily indicative of symptomatic disorders.

The lymphocyte proliferation response to budgerigar serum. The incorporation of ^{3}H-thymidine to lymphocyte cultures stimulated with budgerigar serum of five symptomatic subjects and to those of the three asymptomatic and the seven control subjects are shown in Figs. 1A and 1B. Lymphocytes from symptomatic breeders were significantly stimulated by the addition of budgerigar serum. The optimum dose of budgerigar serum for the lymphocyte stimulation was in the range from 52 mg/ml to 260 mg/ml of serum protein. When 52 mg/ml of budgerigar serum were added to the lymphocyte cultures, the radioactivity counts of Cases 1, 2, 3, 4, and 5 were 19,785±3,738 cpm, 42,302±869 cpm, 77,376±14,162 cpm, 58,594±8,241 cpm, and 13,586±808 cpm, respectively (Fig. 1A). The lymphocytes from the asymptomatic breeders, however, were not stimulated by the addition of budgerigar serum (Fig. 1B), although they also had precipitating antibodies against this antigen. Nor were the lymphocytes from the seven control subjects stimulated by the budgerigar serum.

TABLE 2. *Characteristics of eight subjects studied for lymphocyte reactivity to avian serum*

Subjects	Precipitating antibody titer*		Inhalation challenge test with budgerigar serum†
	Dropping extract	Serum	
Symptomatic breeders			
Case 1	×256	×64	Positive
Case 2	×256	×64	Positive
Case 3	× 64	×32	Positive
Case 4	× 64	×16	Positive
Case 5	× 16	× 8	Positive
Asymptomatic breeders			
Case 6	×128	×64	Negative
Case 7	×128	×64	Negative
Case 8	× 64	× 8	Negative
Normal controls‡	Negative	Negative	n.t.§

* Precipitating antibody titers against budgerigar dropping extracts and budgerigar serum.

† See the text.

‡ They have no experience of breeding any birds.

§ Not tested.

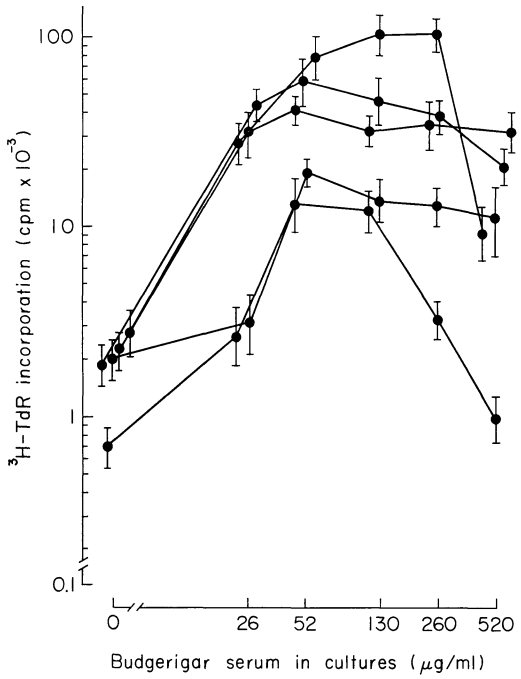

Fig. 1A. Proliferative response of lymphocytes from five symptomatic breeders to budgerigar serum. DNA synthesis measured by incorporated ^{3}H-thymidine in lymphocyte cultures after 7 days of incubation with various concentrations of budgerigar serum. Results are mean±S.E.M. of three cultures.

Evidence for T-cell involvement in the lymphocyte proliferation response to budgerigar serum. To investigate the participation of the T-cell response to budgerigar serum, the PBL of symptomatic breeders were fractionated to T-cells, B-cells, and monocytes. The number of each cell fraction was adjusted to a concentration of 5×10^5 cells/ml. Ninety per cent of the T- or B-cells, and 10% of the monocytes were mixed and incubated in 0.2 ml triplicated samples. Twenty μl of budgerigar serum was added to each cell fraction to give a final concentration of 52 μg budgerigar serum protein/ml. They were then cultured for 7 days, and the effect of these cell conditions on the proliferation response to the budgerigar serum was examined. A most representative sample of data is shown in Fig. 2. The T-cells, depending upon the presence of monocytes, responded to budgerigar antigen in the same way as the PBL's; on the other hand, the B-cells, in spite of the presence of monocytes, responded little to the antigen.

HLA typing. The HLA phenotypes of eight subjects and the phenotype frequencies of HLA antigens in normal Japanese are shown in Table 3. All of the symptomatic breeders had the same A2 antigen and three of them had the B40 antigen. However, the asymptomatic subjects also had high frequencies of A2 and B40 antigens. The haplotype frequency of A2/B40 from 218 normal Japanese

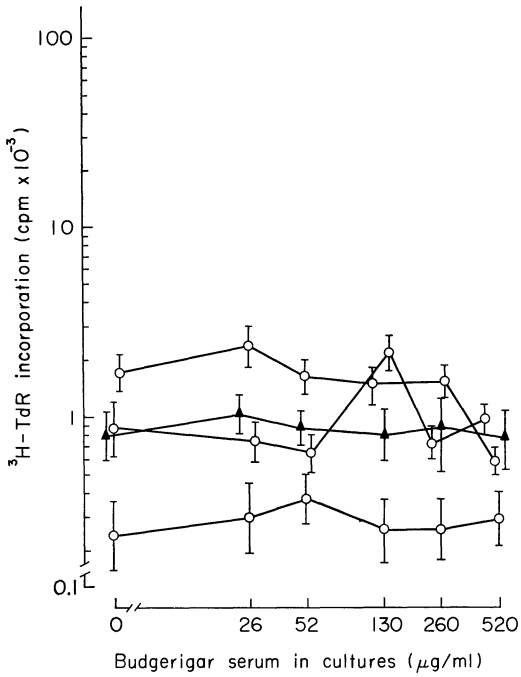

Fig. 1B. Proliferative response of lymphocytes from three asymptomatic breeders (o—o), and seven normal controls (▲—▲). DNA synthesis measured by incorporated ^{3}H-thymidine in lymphocyte cultures after 7 days of incubation with various concentrations of budgerigar serum.

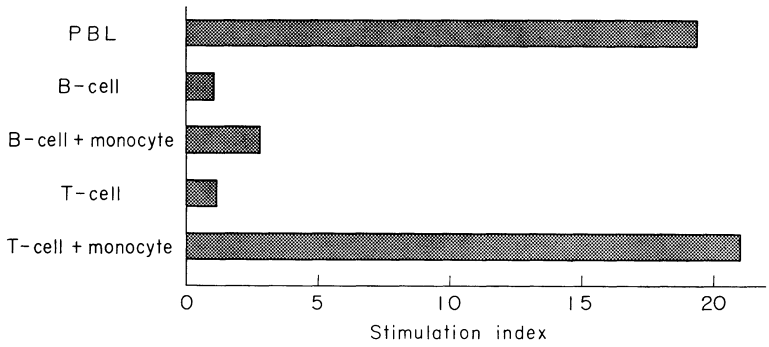

Fig. 2. Evidence for T-cell involvement in the lymphocyte proliferation to budgerigar serum. The results were expressed as a ratio of cpm in presence of antigen to cpm in absence of antigen. The ratio was termed the stimulation index. PBL; peripheral blood lymphocytes.

controls was 63.2±23.5%, and phenotype frequencies of A2 and B40 was 49.0% and 37.6%, respectively.

TABLE 3. *HLA phenotypes of symptomatic and asymptomatic subjects*

Symptomatic subjects	
Case 1	A2, A10. B15, B40.
Case 2	A2, AW24. B7, B40.
Case 3	A2, —. B5, —.
Case 4	A2, A10. BW22, B40.
Case 5	A2, AW19. B5, B12.
Asymptomatic subjects	
Case 6	A10, AW24. BW51, BW54.
Case 7	A2, A10. B40, BW51.
Case 8	A2, AW31. BW16, B40.

Phenotype and haplotype frequencies of HLA-antigens in normal Japanese controls (N=218) is A2=49.0%, B40=37.5%, and A2/B40 haplotype is 63.2±23.5%.

DISCUSSION

Many unsolved problems remain to be elucidated in the pathogenesis of hypersensitivity pneumonitis (Schatz et al. 1977), but a number of studies have provided evidence which suggests a possible pathogenesis of this disease (Christensen et al. 1975; Roberts and Moore 1977; Fink and Salvaggio 1978). Some studies have shown that the cell-mediated immunity response may be closely related to the pathogenesis (Moore et al. 1974; Fink et al. 1975; Schatz et al. 1976b). Experimental animal models (Miyamoto and Kabe 1971; Miyamoto et al. 1971; Peterson et al. 1979) and the histopathology of lung biopsies (Hensely et al. 1969) also supports this opinion.

In this study, we have directly shown that it is the T-cell mediated immunity response that plays the important role in the pathogenesis of bird breeder's lung; i. e., the T-cells of symptomatic breeders, depending upon the presence of monocytes, significantly responded to budgerigar antigen, and showed a high stimulation index. On the other hand, the B-cells, in spite of the presence of monocytes, responded little to the antigen and showed a slightly increased stimulation index. Because the B-cell fraction used here contained 10% T-cells, it might be these T-cells in the sample which responded to the budgerigar antigen.

The results presented here also indicate a significant role of the monocytes in the activation of T-cells by budgerigar serum in vitro. Recent data suggest that the activation of sensitized T-lymphocytes by many antigens and mitogens assessed by lymphocyte proliferation in vitro, involves cooperation between the lymphocytes and the monocytes (Seeger and Oppenheim 1970; Rosenthal and Shevach 1973). Our results correspond to these findings and suggest some possibilities for the pathogenesis of bird breeder's lung. In both symptomatic and asymptomatic breeders, it seems more likely that monocytes would have the ability to present bird antigens not only to B-cells but also to T-cells in an appropriate immunogen form. However, in symptomatic breeders, the T-cells would be more actively sensitized than in the asymptomatic breeders and would be susceptible to stimulation by the etiologic antigen. The presence of sensitized lymphocytes was first

observed in symptomatic but not asymptomatic bird breeders (Hansen and Penny 1974); however, subsequent studies have also identified them in asymptomatic breeders (Schatz et al. 1976a). In the present study, we could not observe the proliferation response of lymphocytes in asymptomatic breeders. However, the apparent separation in the present study between the response of the two groups could well be a reflection of the small number (only three) of the asymptomatic subjects tested. In general, there is only a small percentage of persons suffering from hypersensitivity pneumonitis who are regularly exposed to the etiologic agents (Christensen et al. 1975). This suggests that genetic factors, in addition to environmental exposure, may be involved in the induction of the disease. Indeed, the participation of genetic factors in this disease have been reported by some authors. Allen et al. (1977), Rittner et al. (1975), and Berril and Rood (1977) have reported the prevalence of B-locus antigens to be significantly greater in patients with pigeon breeder's disease. Another possible genetic association in pigeon breeder's disease was reported by Radermecker et al. (1975). However, Moore and co-workers (Moore et al. 1975) reported that there was no detectable association between immunoglobulin allotypes and the development of pigeon breeder's disease. Moreover, Rodey et al. (1979) recently reported that no significant association was found between any of these tested HLA specificities and pigeon breeder's disease.

It is interesting to note, in our study, that not only the symptomatic subjects but also the asymptomatic subjects had a high frequency of HLA-A2 and B40 antigens. However, the Japanese subjects in the control and Japanese people in general have a high frequency of these antigens. Consequently, we could not find any significant association between HLA and this disease.

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