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Triiodothyronine Affects the Phytohemagglutinin to Concanavalin A Proliferative Response Ratio in Sex-Linked Dwarf Chickens[1] (42772)

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Abstract. Euthyroid Cornell K strain and sex-linked dwarf (SLD) strain cockerels (which have abnormally low serum triiodothyronine concentrations) were supplemented with either 0, 0.01, 0.1, or 1.0 ppm of triiodothyronine (T_3) in the diet. Peripheral blood lymphocytes (PBL) from these cockerels were obtained by slow-speed centrifugation (slow-spin-prepared PBL). The proliferative response of these PBL to phytohemagglutinin (PHA) and concanavalin A (Con A) was determined when the chicks were 6, 9, and 12 weeks of age. Con A responsiveness was also determined in 12-week-old cockerels using PBL which were separated on Ficoll (Ficoll-prepared PBL). Using slow-spin-prepared PBL, PHA, and Con A responsiveness increased in both strains with increasing levels of T_3 supplementation. This enhancing effect of T_3 was particularly evident in older cockerels. In 6- and 12-week-old SLD strain cockerels, the PHA:Con A response ratio was significantly ($P < 0.05$) lower than in K strain cockerels. At 12 weeks of age the PHA:Con A response ratio of the SLD strain was elevated to K strain control levels by T_3 supplementation. Therefore, the lower PHA:Con A response ratio in the SLD strain appears to be partially due to the existing peripheral hypothyroidism in this strain. Using Ficoll-prepared PBL, the effects of T_3 on Con A responsiveness differed from those observed when slow-spin-prepared PBL were used. From this study we conclude that T_3 supplementation affects mitogen responsiveness and the PHA:Con A response ratio. However, the effects of T_3 on mitogen responsiveness depend on the age of the chicken, the level of T_3 supplemented, the T cell population stimulated, and the method of lymphocyte enrichment.

Administration of exogenous iodothyronines to euthyroid animals has been reported to increase (1, 2), decrease (3, 4), or have no effect (5, 6) on various aspects of immune function. This lack of agreement in the literature may be due to several factors. For example, the effects of triiodothyronine (T_3) on the proliferative response to concanavalin A (Con A) in chickens were shown to be dose- and age-dependent (2).

In the present study, the effect of exogenously administered T_3 on cell-mediated immunity was examined in K and sex-linked dwarf (SLD) strain chickens. Compared to the control K strain with normal endocrine function, the SLD strain chicken has a number of endocrine abnormalities. Among these is a characteristic depression in serum T_3 concentrations due to a deficiency of 5′-monodeiodinase activity (7). This enzyme is necessary for the peripheral conversion of thyroxine (T_4) to the more biologically active T_3 and is therefore responsible for the formation of most of the T_3 present in the serum. As a result of this deficiency, the SLD strain may be viewed as a suitable model for examining the effects of a naturally occurring form of hypothyroidism on immune function. However, other endocrine imbalances are also known to be present in this strain including abnormal growth hormone (GH) concentrations (7, 8), decreased numbers of tissue GH receptors in older animals (9), and low concentrations of somatomedins (10). Whether all or any of these imbalances are directly related to the iodothyronine levels in this strain remains unclear (11).

One way to assess general cell-mediated immune capabilities is to examine the re-

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sponse of lymphocytes to stimulation with mitogens such as Con A and phytohemagglutinin (PHA). Both PHA and Con A have been shown to be T cell specific in mammalian (12) and avian species (13). In mammalian species, Con A stimulates proliferation of different T cell subpopulations than does PHA (12). Thus, examination of both the PHA and the Con A response in animals supplemented with T_3 provides information about the effects of T_3 on different T cell subpopulations.

Using Ficoll-prepared peripheral blood lymphocytes (PBL), we recently examined the effects of dietary T_3 supplementation on Con A responsiveness in K and SLD strain chickens (2). However, examination of PHA responsiveness using Ficoll-prepared PBL resulted in very low or undetectable levels of proliferation (unpublished results). There is evidence that in chickens different methods of lymphocyte enrichment yield cell preparations which respond differently to mitogen stimulation (14, 15). Particularly, Ficoll-prepared PBL have been shown to exhibit depressed mitogen responsiveness compared to slow-spin-prepared PBL (14).

The purpose of the study reported here was to examine the effect of T_3 supplementation on the PHA:Con A response ratio of slow-spin cell preparations as a means of assessing differential effects of T_3 state on T cell subpopulations.

Materials and Methods. *Experimental animals.* Two Single Comb White Leghorn strains of chickens, developed and maintained at Cornell University, were used in these experiments. These were the normal-growing Cornell K strain and the SLD strain. These strains were developed from related parental stock (16) and have been maintained as closed populations for more than 25 years. Lines within each of these strains that are homozygous *B*15/*B*15 at the major histocompatibility complex (MHC) have been developed by blood typing and pedigree mating over a minimum of four generations (17). One-day-old male chicks from each strain were randomly assigned to treatment groups and provided with feed (Cornell C, a commercially formulated chick starter mash) and water *ad libitum.* Chicks were housed under a 15L:9D lighting regimen in thermostatically controlled battery-brooders with raised wire floors until 6 weeks of age. Thereafter, the birds were transferred to a separate grower facility, fed a commercial grower mash, and maintained on a 9.5L:14.5D lighting regimen.

Experimental design. Twenty-four 1-day-old cockerels from each strain were randomly assigned to each of four treatment groups. Treatments consisted of feeding the cockerels diets supplemented with either 0, 0.01, 0.1, or 1.0 ppm of T_3. Birds were fed the same diet from the day of hatch throughout the duration of the experiment (12 weeks). For the mitogen assays, blood samples from two birds were pooled yielding 12 pools of blood for each strain and treatment group. The proliferative response of lymphocytes to the mitogens Con A and PHA were assayed at 6, 9, and 12 weeks of age. Blood from the same two birds was pooled for each assay.

Hormone treatments. The effectiveness of dietary iodothyronine supplementation has previously been demonstrated (18) and is routinely used in our laboratory (2, 11, 19). In this study, the diet was supplemented with T_3 (3,3′, 5-triiodo-L-thyronine, Sigma Chemical Co.) as described by Erf and Marsh (2).

Culture medium. Commercial RPMI 1640 medium was used containing 25 m*M* Hepes buffer (GIBCO, Laboratories) and supplemented with 1 ml of 5×10^{-3} *M* 2-mercaptoethanol, 1 ml of 200 m*M* L-glutamine, 2 ml of commercial heat inactivated chicken serum, 1 ml of Pen-Strep (10,000 U penicillin and 10 mg streptomycin), and 1 ml of 0.25 mg/ml fungizone per 100 ml of RPMI medium. Medium supplemented in this way is hereafter referred to as "complete" medium.

Lymphocyte preparations. At 6, 9, and 12 weeks of age, 2.4 ml of blood was drawn from the wing vein of each bird into syringes containing 0.6 ml of RPMI 1640 with 10 USP units of heparin/ml (Heparin Sodium, LyphoMed). To have samples large enough to carry out all assays, blood samples from two birds were pooled in 16×100-mm culture tubes and PBL were obtained by slow-speed centrifugation at 80*g* for 5 min (14). The plasma containing PBL was removed and slow-spin PBL were washed twice in phosphate-buffered saline (PBS, 0.75% saline, pH 7.2), at 200*g* for 10 min. Cell pellets were resuspended in complete medium. Cell concentrations in all slow-spin cell preparations were adjusted to 1×10^7 viable PBL/ml of complete medium using the method described by Erf and Marsh (2).

Heparinized blood from 12-week-old cockerels was also used for Ficoll cell preparations. Blood samples were carefully layered onto Ficoll (Ficoll–Paque, Pharmacia Inc.) and centrifuged at 400*g* for 30 min. Peripheral mononuclear cells were removed from the Ficoll-plasma interphase, washed twice in PBS, and resuspended in complete medium. Because Ficoll cell preparations consist of PBL, monocytes, and large numbers of thrombocytes (14), cell suspensions were adjusted to 1×10^7 PBL/ml. This was done by visual discrimination of lymphocytes from other peripheral mononuclear cells using the method described by Erf and Marsh (2).

Cell proliferation assays. To assess PHA responsiveness, 0.1 ml of each slow-spin cell preparation (1×10^6 PBL/well) was added to 6 wells of a 96-well U-bottom microtiter plate. To three of these wells, 0.1 ml of complete medium was added (unstimulated controls), and to the other three wells 30 μg of PHA/well (PHA-P, E-Y Laboratories, San Mateo, CA) in 0.1 ml of complete medium was added (stimulated cells). Thirty micrograms of PHA/well has previously been determined to yield optimal levels during lymphocyte proliferation in our assay system. Using this dose of PHA, lymphocyte proliferation peaked during the last 24 hr of a 42 hr culture. To determine lymphocyte proliferation, [^{3}H]thymidine (1.5 μCi/0.05 ml of complete medium) was added to each culture after 18 hr of incubation. Cells were incubated for another 24 hr and then harvested onto glass-fiber filters. The uptake of [^{3}H]thymidine was determined by scintillation counting as counts per minute (cpm) and the data were expressed as the change in cpm (i.e., cpm of stimulated culture − cpm of unstimulated cultures). Proliferation of the unstimulated cultures always yielded less than 1000 cpm and usually ranged between 300 and 600 cpm.

For the Con A assays, optimal mitogen dosage and time of peak lymphocyte proliferation were also determined previously for all strain and treatment groups. The Con A assays were conducted using the same procedure as in the PHA assays. In these assays, however, cultures were stimulated with 5 μg Con A (Sigma Chemical Co., St. Louis, MO) in 0.1 ml complete medium per well. Tritiated-thymidine was added to all Con A cultures after 36 hr of incubation in the same manner as described for the PHA assay.

Lymphocyte:monocyte ratio. For each strain and treatment combination, 9 blood smears/group were prepared to determine the ratio of PBL and monocytes in the Ficoll cell preparations. Twenty microliters of the Ficoll cell preparations was placed on glass slides and evenly spread over the surface. After drying, slides were fixed in methanol and stained with Wright stain. The ratio of PBL to monocytes was determined after counting a total of 300 mononuclear leucocytes per slide.

Statistical analysis. Data were analyzed for main effects of strain and treatment and strain by treatment interactions using two-way analysis of variance (ANOVA). Single effects due to strain and treatment were determined using the protected least significant difference test and linear regression analysis (20). All PHA and Con A data were normalized by square root transformation prior to statistical analysis. In all statistical tests the level of significance was chosen to be $P < 0.05$. Two-way analysis of variance revealed no significant interactions between treatment and strain effects in all mitogen assays. For this reason, primarily the main treatment effects are reported and discussed for both strains. However, in graphical presentations of the data (Figs. 1–4), single effects of treatments are shown for each strain.

Results. The ratio of lymphocytes to monocytes present in the Ficoll cell preparations was unaffected by strain or dietary T_3 supplementation. The average lymphocyte to monocyte ratio in these cell preparations was 6.4 ± 0.5 and 5.9 ± 0.6 (mean ratio $\pm$ SEM) for K strain and SLD strain chickens, respectively.

Effects of dietary T_3 supplementation on the PHA responsiveness of slow-spin-prepared PBL from K and SLD strain cockerels were age- and dose-dependent (Fig. 1). In

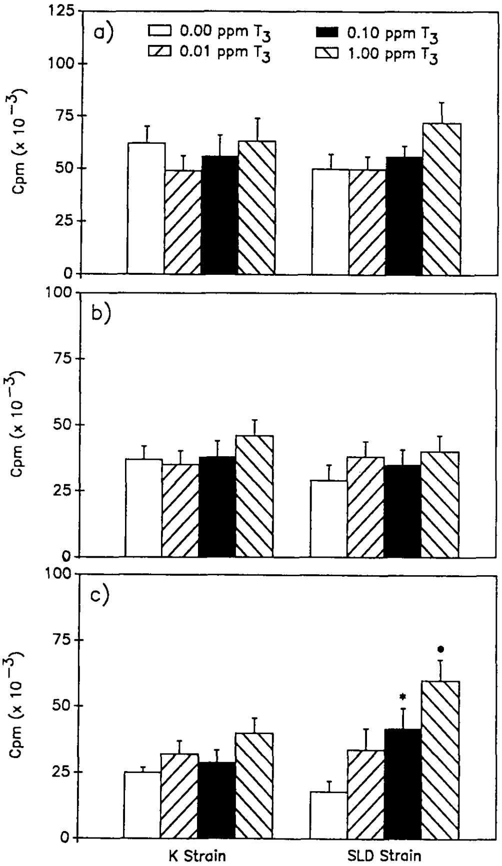

FIG. 1. The proliferative response of PHA-stimulated lymphocytes isolated from whole blood by slow-speed centrifugation. PHA responsiveness was examined in 6- (a), 9- (b), and 12-week-old (c) K and SLD strain cockerels receiving diets supplemented with 0, 0.01, 0.1, and 1.0 ppm T_3 since the day of hatch. Each bar represents the mean change in counts per minute of 12 samples $\pm$ SEM. The asterisk indicates significant ($P \leqslant 0.05$) single treatment effects compared to the untreated control of that strain and age group.

most age and strain groups the higher concentrations of T_3 tended to increase PHA responsiveness (Fig. 1a–c). The main effect of T_3 treatment was, however, only significant in 12-week-old cockerels (Fig. 1c). Although there were no significant main strain effects in PHA responsiveness between SLD and K strain cockerels at all ages, graphical representation of the data (Fig. 1) suggests that the SLD strain is somewhat more responsive than the K strain.

At 6 weeks of age there was no significant treatment effect on the Con A responsiveness of slow-spin-prepared PBL (Fig. 2a). In 9- and 12-week-old cockerels there was a significant main treatment effect, whereby T_3 supplementation resulted in enhanced Con A responsiveness. In 9-week-old cockerels of both strains, linear regression analysis showed that Con A responsiveness increased directly with increasing levels of T_3 supplementation (Fig. 2b and 2c). In all age groups, there was a significant main strain effect on Con A responsiveness of slow-spin-prepared PBL, with the SLD strain exhibiting greater

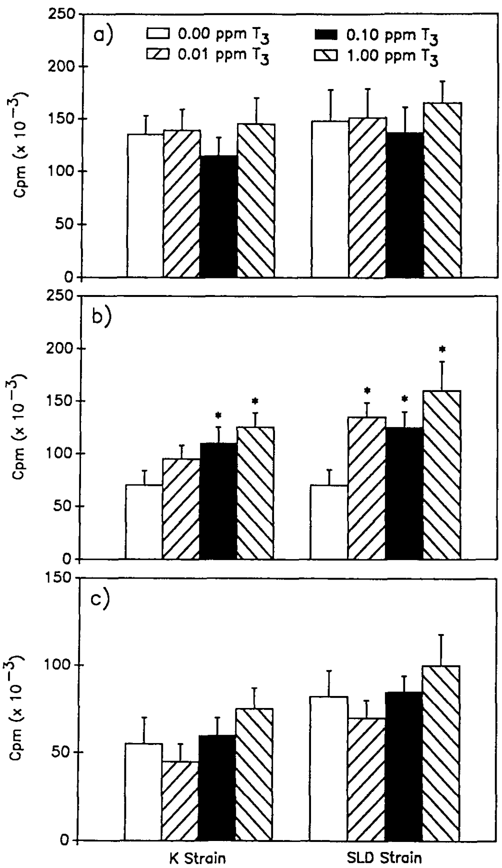

FIG. 2. The proliferative response of Con A-stimulated lymphocytes isolated from whole blood by slow-speed centrifugation. Con A responsiveness was examined in 6- (a), 9- (b), and 12-week-old (c) K and SLD strain cockerels receiving diets supplemented with 0, 0.01, 0.1, and 1.0 ppm T_3 since the day of hatch. Each bar represents the mean change in counts per minute of 12 samples $\pm$ SEM. The asterisk indicates significant ($P \leqslant 0.05$) single treatment effects compared to the untreated control of that strain and age group.

Con A responsiveness than the K strain cockerels.

The observed Con A response pattern of Ficoll-prepared PBL from T_3-treated, 12-week-old K and SLD strain cockerels (Fig. 3) was in agreement with that observed in an earlier study by Erf and Marsh (2) and in direct contrast to the response observed using slow-spin cell preparations. Although treatment effects were not statistically significant, supplementation with 0.01 ppm of T_3 resulted in some enhancement of the proliferative response of Ficoll-prepared PBL whereas higher levels of T_3 supplementation did not, i.e., there was no direct effect on PBL proliferation with increasing dosage as was seen in the previous experiment using slow-spin-prepared lymphocytes.

There was a significant main strain difference in Con A responsiveness of Ficoll-prepared PBL. However, contrary to the slow-spin-prepared PBL but like our observation in the earlier study (2), Ficoll-prepared PBL from SLD strain cockerels exhibited an overall lower Con A response than Ficoll-prepared PBL from K strain cockerels.

The PHA:Con A response ratio of slow-spin-prepared PBL was not significantly affected by dietary T_3 supplementation in 6- and 9-week-old cockerels (Fig. 4a and 4b). In 12-week-old cockerels there was a significant interaction between treatment and strain effects. Thus, the two strains were analyzed separately. Supplementation of 12-week-old

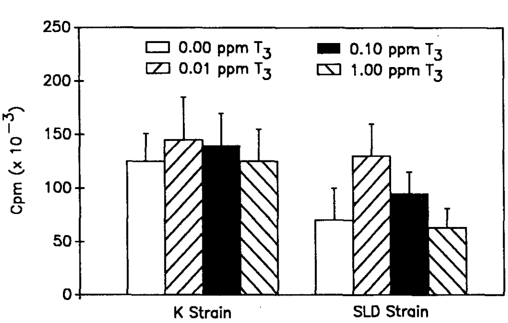

FIG. 3. The proliferative response of Con A-stimulated lymphocytes isolated from whole blood by floatation on Ficoll. Con A responsiveness was examined in 12-week-old K and SLD strain cockerels receiving diets supplemented with 0, 0.01, 0.1, and 1.0 ppm T_3 since the day of hatch. Each bar represents the mean change in counts per minute of 12 samples ± SEM.

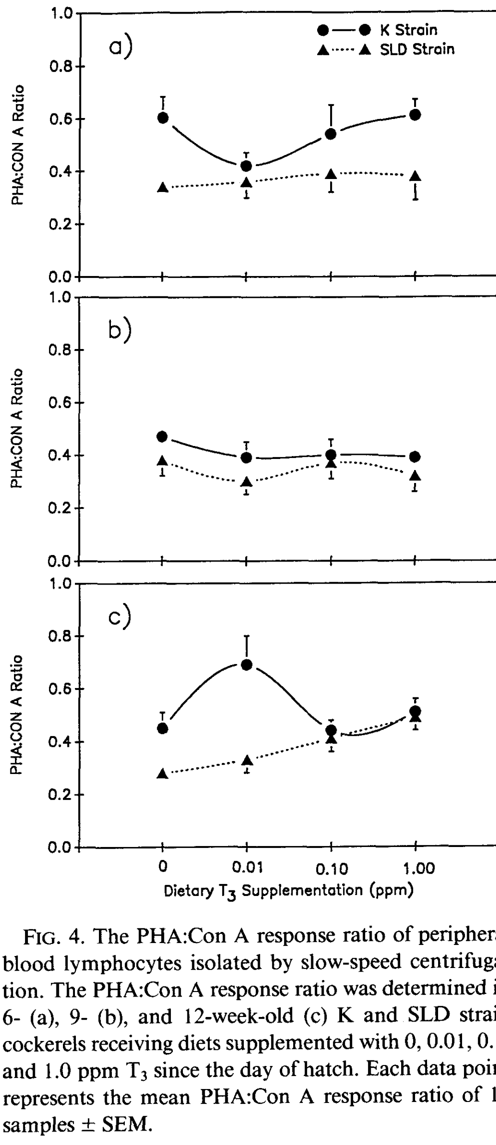

FIG. 4. The PHA:Con A response ratio of peripheral blood lymphocytes isolated by slow-speed centrifugation. The PHA:Con A response ratio was determined in 6- (a), 9- (b), and 12-week-old (c) K and SLD strain cockerels receiving diets supplemented with 0, 0.01, 0.1, and 1.0 ppm T_3 since the day of hatch. Each data point represents the mean PHA:Con A response ratio of 12 samples ± SEM.

K strain cockerels with 0.01 ppm of T_3 significantly enhanced the PHA:Con A response ratio. With higher doses of T_3 supplementation, the PHA:Con A response ratio of 12-week-old K strain cockerels returned to the unsupplemented control levels (Fig. 4c). However, the PHA:Con A response ratio increased significantly in 12-week-old SLD cockerels with increasing levels of T_3 supplementation (Fig. 4c). The PHA:Con A response ratio of the SLD strain was generally lower than that of the K strain cockerels at all ages. A significant difference between strains in the form of a main strain effect was observed in both 6- and 12-week-old cockerels (Fig. 4).

Discussion. Assessment of the Con A and PHA responsiveness of T lymphocytes serves as an indicator for general cell-mediated immune capabilities and is often used as a diagnostic tool in the detection of abnormal cell-mediated immune function (12). In mammalian species, PHA is known to stimulate proliferation of different T cell subpopulations than are stimulated by Con A. The PHA:Con A response ratio has been used as an indicator for assessing the normal balance of T cell subpopulations (21). In the present study, PHA and Con A responsiveness was assessed to examine the relationship between iodothyronine state and T cell mitogenic responsiveness in K and SLD strain chickens.

The enhancing effects of dietary T_3 supplementation on the PHA and Con A response of slow-spin-prepared PBL were demonstrated in both strains, especially in older cockerels. Similarly, the PHA:Con A response ratio was not significantly affected by T_3 supplementation until the cockerels were 12 weeks old. At this age, the PHA:Con A response ratio in the SLD strain cockerels increased with increasing levels of T_3 supplementation. In the K strain cockerels only the lowest level of T_3 supplementation significantly enhanced the PHA:Con A response ratio, with higher T_3 levels having no effect. Interestingly, plasma T_3 concentrations (Marsh and Erf, submitted for publication) in the SLD strain were not only significantly reduced in the unsupplemented 12-week-old SLD strain cockerels (0.4 ng/ml) compared to K strain cockerels (1.25 ng/ml), but also in those SLD strain cockerels which were supplemented with 1.0 ppm T_3 (5.9 ng/ml in SLD strain versus 10.6 ng/ml in the K strain). It is possible that in the functionally hypothyroid SLD strain chicken, T_3 supplementation resulted in normal plasma T_3 concentrations which enhanced the PHA:Con A response ratio to the level of the K control chicks. Supplementation of the euthyroid K strain cockerels with already normal concentrations of T_3, however, may have resulted in a hyperthyroid condition with T_3 concentrations too high to have enhancing effects on the PHA:Con A ratio.

Generally, the PHA:Con A response ratio varied depending on the strain of chickens and the dose of T_3 supplementation. Thus it would appear that in the chicken, like in mammals, PHA and Con A may stimulate different T cell subpopulations. Assuming this is the case, the functionally hypothyroid SLD strain cockerels may have an abnormal balance among T cell subsets which can be manipulated with T_3 supplementation. This is in agreement with the observation by Fabris *et al.* (21) that the low PHA:Con A response ratio in old mice compared to young mice can be significantly enhanced by short-term treatment with thyroxine. Further evidence that iodothyronine state can affect the normal balance between T cell subpopulations was reported by Pacini *et al.* (22). They observed that when compared to normal rats, hypothyroid (thyroidectomized or 6-propyl-2-thiouracil treated) rats had an increased proportion of suppressor T cells in the blood resulting in a decreased helper:suppressor T cell ratio.

In the present study the presence or absence of either strain or treatment effects on the Con A response depended on the method of lymphocyte enrichment used. Using slow-spin cell preparations, SLD strain cockerels exhibited an overall greater Con A responsiveness than K strain cockerels. Furthermore, higher levels of T_3 supplementation increased the Con A response of slow-spin cell preparations in both strains. However, the opposite results were obtained when Con A responsiveness was examined in the same strain and treatment groups using Ficoll cell preparations. In this case, like in an earlier report (2), SLD strain cockerels exhibited a lower Con A response than those of the K strain. Furthermore, higher levels of T_3 supplementation depressed the Con A responsiveness of Ficoll cell preparations in both strains. Using chickens, it has been established that Ficoll and slow-spin cell preparations differ in their immune cell compositions. As shown by Schaefer *et al.* (14), slow-spin cell preparations consisted almost entirely of lymphocytes with <0.1% monocytes whereas Ficoll cell preparations consisted of PBL, 6 to 8% monocytes, and a large number of thrombocytes.

We have made similar observations regarding the cell compositions of slow-spin and Ficoll preparations (unpublished observation). Since thrombocytes do not appear to affect the Con A response (15), they would seem to be of negligible consequence in these studies. However, substantial evidence exists from work done in chickens that the monocytes present in Ficoll cell preparations may exert a suppressive effect on the Con A response (14). In the present study, unlike the observations by Schaefer *et al.* (14), the Con A response of Ficoll cell preparations was not noticeably suppressed when compared to the response of the slow-spin cell preparations. However, a major difference was found between the two different cell preparations in the PHA response (unpublished observations) with slow-spin cell preparations giving a much greater proliferative response to PHA than was obtained using Ficoll-prepared cells.

On the assumption that the presence or absence of monocyte suppression is the only difference between Ficoll and slow-spin cell preparations several hypotheses can be put forward. First, the number of monocytes and hence monocyte suppression, may vary depending on the strain and/or treatment. This hypothesis can be readily disputed by the fact that the monocyte:lymphocyte ratio in the Ficoll cell preparations was the same in both strains and did not change with T_3 supplementation. Second, the suppressive activity of monocytes may differ depending on strain and/or treatment. If this is true, it would appear that the suppressive activity of Ficoll-prepared monocytes is higher in SLD strain than in K strain cockerels. This is based on the fact that slow-spin preparations from the SLD strain had an overall greater Con A response than the K strain, whereas the reverse relationship existed when Ficoll cell preparations were stimulated with Con A. Furthermore, if the suppressive activities in the Ficoll cell preparations from all treatment groups were constant, one would expect the Con A response of Ficoll-prepared cells to be lower compared to slow-spin-prepared cells but the effects of T_3 should be similar with both types of cell preparations. However, high levels of T_3 supplementation enhanced the Con A response of slow-spin cell preparations and depressed the Con A response of Ficoll preparations. This suggests that in Ficoll cell preparations the suppressive activity of monocytes may increase with increasing levels of T_3 supplementation. As reported by Schaefer *et al.* (14) the monocytes present in Ficoll cell preparation appear to mediate their suppressive effects by soluble factors.

Finally, an alternative hypothesis is that other factors are involved in the regulation of the Con A response in the two types of cell preparations. For example, the two methods of lymphocyte enrichment may have yielded different T cell subpopulations which respond differently to mitogenic stimulation and to T_3 supplementation. Given the differential responsiveness of these two preparations to Con A and PHA, this would seem to be a viable hypothesis. Further studies are required to examine the above possibilities.

Several conclusions can be drawn from this study. Clearly, T_3 does have enhancing effects on both PHA and Con A responsiveness in K and SLD strain cockerels. Generally, the effects of T_3 were dose-dependent and were more prominent in 9- and 12-week-old cockerels. At these ages, mitogen responsiveness tended to increase with increasing doses of T_3 supplementation. The PHA:Con A response ratio was lower in the SLD strain than in the K strain cockerels at all ages. In 12-week-old SLD strain cockerels, the lower PHA:Con A response ratio increased to the levels of the K strain control as a result of T_3 supplementation. Thus the SLD strains' depressed PHA:Con A response ratio may be due to this strains' peripheral hypothyroidism. Based on the observed changes in the PHA:Con A ratio depending on strain and treatment, it appears that in the chicken, like in mammals, PHA and Con A may stimulate different T cell subpopulations. Finally, we have shown that the effect of T_3 on Con A and PHA responsiveness can differ dramatically depending on the method of lymphocyte enrichment. This is further evidence for the differential effectiveness of T_3 depending on the immune cell populations. Different T cell subpopulations may be present in the Ficoll than in the slow-spin cell preparations which differ in their response to Con A stimulation and T_3 supplementation. On the basis of these data, however, one cannot rule out the level of suppressive monocyte activity present in the Ficoll cell preparations varying as a result of the strain of cockerels and the level of T_3 supplementation.

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