AFRICAN JOURNAL OF MEDICINE and medical sciences

VOLUME 30, NUMBER 4, DECEMBER 2001

EDITOR:
B. O. OSOTIMEHIN

ASSISTANT EDITOR:
A. O. UWAIFO

ISSN 1116 — 4077

Afr. J. Med. med. Sci. (2001) **30**, 255-259

T-lymphocyte subsets in patients with hookworm infection in Zaria, Nigeria

GC Onyemelukwe and BOP Musa
Immunology Unit, Department of Medicine, Ahmadu Bello University, Zaria, Nigeria

Summary
Peripheral lymphocyte subsets CD3, CD4, CD8 were studied using monoclonal antibodies to determine the mechanism of immunosuppression observed in an earlier study with total T-cells using the sheep erythrocyte rosetting technique. The study was carried out in 37 Nigerian patients with hookworm infection (*Necator americanus* and *Ancylostoma duodenale* and 17 healthy Nigerians as controls. The leucocyte migration inhibition (LMI) test was also carried out to assess the functional integrity of the lymphocytes, while the hookworm status of the patients was assessed by the Stoll technique for measuring egg counts. The results of the T-cell studied showed that CD3 and CD4 cell percentages were significantly depressed in hookworm patients compared to controls ($P<0.05$). The difference between mean CD8 percentages and absolutes in hookworms patients and controls were not significant. Absolute CD3 and CD4 numbers were not significantly lowered compared to controls in the study and CD4/CD8 ratios were insignificantly reduced. Patients with polyparasitism including hookworm infection showed significant reduction in CD3 and CD4 cells when compared with controls and with patients with hookworm infection alone. The leucocyte migration inhibition response to purified protein derivative of *M. tuberculosis* (PPD) was significantly decreased in hookworm patients compared to controls, confirming that functionally cell mediated immunity is depressed in hookworm infection.

Keywords: *Hookworms, lymphocytes, MIF, aneamia, CMI, WBC*

Resume
Les sous-ensemble lymphocytes peripheri ques CD3, CD4, CD8 ont ete etudies utilisant les anticorps monoclonles pour determiner le mechanisme de l'immuno-suppression observe dans une premiere etude avec les cellules T par la technique rosette erythrocyte du monton. 37 patients Nigerians con ete examines pour l'infection bloquee dee vers (Necator Americamis et Ancylostoma duodemale) et 17 Nigerian controle en bonne sante. Le taux d'inhibition migratoire de leucocyte (IML) a ete examine pour estimer l'integrite fonctionelle des lymphocytes, alors que le statut des vers a ete examine par la technique de stoll pour le comptage des eufs. Les resultats des cellul;es T. etudies ont montre que le pourcentage des cellules CD3 et CD4 etait tres peu significatif chez les patients infects compare au controle ($P < 0.05$). La difference entre le pourcentage moyen de CD8 et les absolus chez les patients et ceux de controle n'etait pas significative le nombre d;absolu CD3 et CD4 n'etait pas bas compare au controle dans l'etuide et les proportions CD4/CD8 etaient reduit de fagon insignifiable. Les maladies avec polyparasitisme incllant l'infection aux vers ont montre une reduction significative en cellules CD3 et CD4 compare avec les controles et avec les patients infectes des vers setlement. La reponse de l'inhibition migratoire des leucocytes pour purifier la derivee proteinnique de la tuberculose M (PDP) a baisse significativement chez les patients infects des vers, compare a ceux des controles, confirment aussi que l'immunite fonctionelle des cellules est faible chez les individus infectes par les vers.

Correspondence: Professor G.C. Onyemelukwe, Department of Medicine, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria.

Introduction

Hookworms like most of the established adult worms or nematode parasites of man persist in the lumen of patients despite intact immune defence mechanisms and this persistence is often for a very long period of time [1]. There have been speculations that possibly the hookworms avoid the action of the immune response by some of "adaptation"[2], by the physical adherence by means of hooks to the intestinal epithelia, by the secretion of acetylcholinesterase which acts as a "biological holdfast" [3], and by the indiscriminate induction of reaginic antibody production[4]. Little has been studied about the quantity of immunocompetent cells in the blood of patients with hookworm infection. It is known that while there are Ag-specific cells in the gut, these cells after activation enter the lymphatics and pass some time on the peripheral circulation before returning to the gut. In an earlier study [5], the level of sheep erythrocyte rossette forming T-cells were significantly lowered in hookworm patients compared to controls. This was said to be contributory to the persistence of hookworm in man, especially in those heavily infected. Consequently this study was undertaken to further examine the role of the cell-mediated immune system in hookworm infection, by evaluating lymphocyte subsets in hookworm patients and controls.

Materials and methods

The study group comprised 37 patients either attending the out-patient Department or admitted in one of the wards of the Ahmadu Bello University Teaching Hospital, Zaria. The controls numbering seventeen [17] were healthy Nigerian volunteers within the age brackets of the patients. Hookworm and any other worm infection was excluded from the controls by the absence of the hookworm or other ova in their stools. Malaria and any other disease requiring the use of antibiotics were also excluded. Routine haematological differential films were also used to ascertain a normal blood film. The age of the patients ranged between 14 and 50 years with a mean of 31 ? 14 years while the controls had an age range of 16 to 45 years with a mean of 29?7 years. There were 20 male and 13 female patients and 11 male and 6 female controls.

Stool examination:
This was carried out as described in our earlier study [5]. The direct smear method [6] was used to examine for ova, while the egg counting technique [7] and the species identification method [8] were used to determine worm load and identify hookworm species, respectively.

Peripheral blood specimens
A 17ml volume of blood was drawn by venepuncture from each individual. An aliquot of 10ml was mixed with 100 units of heparin for T-lymphocyte assays. A second aliquot of 5ml was allowed to clot at room temperature for about 30 minutes for the

separation of serum, while another 2 ml of blood was dispensed into an EDTA bottle for haematological assessment.

Peripheral blood cell counts
Standard haematological techniques were used to obtain total white blood cell (WBC) and differential white blood cell counts on each of the EDTA blood specimens [9].

Cell Separations:
Peripheral blood lymphocytes (PBL) were isolated by the Ficoll-Hypaque density gradient centrifugation [10] and washed twice in Eagles Minimum Essential Medium (MEM) supplemented with 4.4% sodium bicarbonate for pH adjustment (Flow Laboratories, Bonn, FRG). Purification of the resultant lymphocyte population was verified by the inclusion of a differential count on the mononuclear cell interface harvested after layering on Ficoll-Hypaque (Sigma Chemical Co. USA).

Enumeration of lymphocytes by monoclonal antibodies
Lymphocytes subpopulations were characterized by staining with monoclonal antibodies of the anti-Leu series conjugated with fluorescein (Becton-Dickinson Immunocytochemistry system, California). The pan T-cell antibody, anti Leu 4 recognizes peripheral human T-cells (CD3), anti Leu 3a (CD4) defines the helper/inducer subset and anti leu 2a recognizes the antigen (CD8) on T-suppressor/cytotoxic cells. The staining procedure was as outlined in the Becton-Dickinson Source book for standard immunological assays. A 50il aliquot of the lymphocyte suspension separated above was placed in a small plastic tube and 20il of the appropriate monoclonal antibody added. This was incubated for 30 minutes in crushed ice at 4°C after which it was washed twice in 50il MEM. The cells were finally resuspended in 40il of MEM and a drop of this suspension placed on a glass slide, was covered with a coverslip and sealed with nail varnish. This was kept at 4°C for 15 minutes for the cells to settle down and the fluorescence read using a ZEISS epi 14 fluorescent microscope by screening random fields until a total of 200 lymphocytes (both positive and negative) were counted. The number of positive cells was then expressed as percentage of the total count and the absolute number of the cells was calculated out from the total and differential white blood cell (WBC) counts.

Leucocyte Migration Inhibition Test (LMIT)
The method described by Rosenberg [11] and outline in our earlier study [5] was employed to determine leucocyte migration inhibition response to purified protein derivative (PPD). Briefly, an 8ml aliquot of heparinized blood was collected from both patients and controls and allowed to stand for about an hour at 37°C to permit sedimentation of red blood cells. The supernatant rich in leucocytes was removed and washed three times with Eagles Minimum Essential Medium (MEM) for 10 minutes at 1,800rpm. The white blood cells were then resuspended in 1ml RPMI 1640 containing 10% foetal calf serum and antibiotics, drawn into capillary tubes with plasticine to seal and centrifuged at 1,800 rpm for 10 minutes. The capillary tubes were then cut at the cell-fluid interface and placed in small plastic chambers containing culture medium with PPD enriched with 10% foetal calf-serum and penicillin, streptomycin. The chambers were sealed with coverslips and incubated at 37°C in CO_2 for 2 hours. The resulting migration areas were then projected onto paper, outlined and the area measured. The migration inhibition percentage (MI) was calculated as follows:

$$\% MI = \frac{1 - \text{area with antigen}}{\text{area without antigen}} \times 100$$

Statistical Analysis: of the difference between means was done using the student t-test.

Results

Parasitological Findings
The patients were diagnosed to be suffering from hookworm infection based on the recovery of hookworm eggs in their stools. The intensity of infection as indicated by this egg excretion in stool ranged from 100 - 2900 eggs per gram of stool (mean: 800 ova per gram of stool). From the test-tube filter paper culture results, *Necator americanus* was the predominant hookworm species occurring alone in 26 patients (78% of 37 samples) and together with *Ancylostoma duodenale* in 4 patients, (12%). Hookworm was associated with other parasites as follows; *Trichuris trichiura (3%), Strongyloides stercoralis (3%), Ascaris lumbricoides (3%), Schistosoma mansoni (3%), Giardia lamblia (3%)* and *Entamoeba histolytica* (3%).

Table 1: White Blood Cell (WBC) Counts, T-Cell subsets and Leucocyte Migration (LMI) percentages in hookworm patients and controls.

	Hookworm Patients n = 37	Control n = 17	P. values
White Blood Cell counts (WBC)			
Mean WBC x 10^9/L	4.31±1.3	3.33±1.3	
Range	1.2 - 8.0	1.2 - 8.1	P > 0.01
Packed Cell Volume (PCV)			
Mean PCV	33 ± 11	42 ± 4	
Range	11 - 48	32 - 48	P< 0.001
Pan T (CD3 +) Cells			
% mean ± SE	47.8 ± 8.6	59 ± 66	
--> (range)	(7 - 65%)	(49-69%)	P < 0.05
Mean absolute no x 10^9/L±SD	1.12 ± 0.81	0.91±0.24	
(range)	(0.05-4.32)	(0.48-1.89)	P > 0.01
AntiLeu 3a (CD4+) Cells			
% mean ± SE	25.3 ± 6	32 ± 4	
..> (range)	(10 -34)	(25-37)	P > 0.05
Mean absolute no x 10^9/L±SD	0.59±0.45	0.50±0.23	
(range)	(0.02-2.10)	(0.25-0.98)	P > 0.01
AntiLeu 2a (CD8 +) Cells			
% mean ± SE	21± 5	23±14	
...> (range)	(11-39)	(14-29)	P > 0.05
Mean absolute nox10^9/L±SD	0.46±0.35	0.34±0.18	
(range)	(0.02-1.45)	(0.15-0.76)	P > 0.05
0.15 LMI with PPD (%)	34 ± 17	56 ± 6	
(range)	(3-60)	(48-69)	P < 0.005

Haematological Findings
The thin blood films consistently showed hypochromic anaemia in 49% of the cases. The mean haemoglobin level for the patients was 8.3g/dl ? 2.96 while the mean packed cell volume was 33?11. On the basis of haemoglobin levels and degree of hypochromia, the hookworm patients were divided into three groups as follows: Group I with marked anaemia (Hb 1-7 g/dl hypochromia +++); Group II with mild to moderate anaemia (Hb 8-11 g/dl, hypochromia ++) and Group III, patients with no anaemia. A mild degree of eosinophilia (1-6%), was observed in 77% of the cases. Only in 16% of the patients was marked eosinophilia demonstrated (>6%). Mean absolute number of eosinophils (0.36?0.30 x 10^9/L) was also significantly higher ($P<0.05$) when compared with controls (0.06?0.04 x 10^9/L). Mean white blood cell counts for patients and controls were not significantly different ($P>0.05$).

T-cells and Subsets
Mean percentages of CD3 and CD4 cells were significantly lower in all of the hookworm subgroups compared to control

Table 2: T-Cell Subsets and Leucocyte Migration percentages in Hookworm Subgroups and Controls

	Group1 Hookworm with marked anaemia n = 11	Group 2 Hookworm patients with mild moderate anaemia n = 10	Group 3 Hookworm patient with no anaemia n = 16	Control n = 17	P. value
Pan T (CD3 +) Cells					
% mean ± SE	45.3±9.1	50.2 ± 9.9	47.9 ± 6.7	5.9±6.6	
range	(27-61%)	(31-65%)	(31-60%)	(49-69%)	P < 0.05
abs. value x 109/L mean	1.12 ± 0.9	1.27 ± 1.1	0.97 ± 0.43	0.91±0.24	
± SD range	(0.31-3.48)	(0.33-4.32)	(0.65-4.23)	(0.48-1.89)	NS
CD4 + Cel;ls					
% mean ± SD range	22 ± 6	28 ± 6	26 ± 5	32 ± 4	
abs. nos x 109/L ± SD	(10-29)	(19-37)	(15-34)	(25-37)	P < 0.05
range	0.56 ± 0.55	0.69 ± 0.54	0.52 ± 0.27	0.50 ± 0.23	
	(0.17-2.03)	(0.20-2.10)	(0.02-1.02)	(0.23-0.98)	NS
CD8 + Cells					
% mean ± SD range	21 ± 5	21 ± 5	21 ± 6	23 ± 4	
abs. no x 109/L ± SD	(11-29)	(12-29)	(14-37)	(14-29)	NS
	0.48 ± 0.32	0.51 ± 0.36	0.40 ± 0.19	0.34 ± 0.18	
	(0.13-1.23)	(0.13-1.43)	(0.02-0.79)	(0.15-0.78)	NS
LMI (%)	30 ± 20	32 ± 14	41 ± 16	56 ± 6	P < 0.05
	(3-57)	(13-15%)	(11-60%)	(48-69%)	

NS - refers to non-statistically significant difference

Table 3: T-Cell Subsets and Leucocyte Migration Percentages in Hookworm Patients with Associated Parasites and Control

	Patients with Hookworms only n = 26	Patients with Hookworm and other parasites n = 11	Total Hookworm Patients n = 37	Control n = 17	P. value
Pan T (CD3 +) Cells					
% mean ± SD range	49.2 ± 8.0	44.5 ± 9.4	48 ± 9.0	59 ± 6.6	
	(31-65%)	(27-59)	(27-65)	(49-69%)	P < 0.05
Mean abs. no.±SD range	1.21 ± 0.85	0.77 ± 0.41	1.08 ± 0.8	0.91 ± 0.24	
	(0.05-4-23)	(0.31-1.50)	(0.31-4.23)	(0.48-1.89)	NS
CD4 + Cell.					
% mean ± SD range	25 ± 6	22 ± 6	25 ± 6.0	32 ± 4	
	(15-37)	(10-28)	(10-37)	(25-37)	P < 0.05
Mean abs. no.±SD range	0.54 ± 0.47	0.39 ± 0.21	0.60 ± 0.4	0.50 ± 0.23	
	(0.02-2.10)	(0.17-0.76)	(0.17.-2.10)	(0.23-0.98	NS
CD8 + Cells					
% mean ± SD range	22 ± 4	19 ± 8	21 ± 5.0	23 ± 4	
	(16-29)	(11-37)	(11-37)	(14-29)	NS
Mean abs. no. ± SD range	0.50 ± 0.39	0.32 ± 0.26	0.41 ± 0.2	0.34 ± 0.18	
	(0.02-1.43)	(0.12-0.79)	(0.12-1.43)	(0.15-0.78)	NS
LMI (%)	35 ± 18	37 ± 13	35 ± 16	56 ± 6	P < 0.05
	(3-59)	(22-57)	(5-59)	(48-69)	

(P<0.05). There was no significant difference between mean percentages of CD8 cells in patients and controls. Furthermore, the absolute numbers of CD3, CD4 and CD8 were not significantly different between patients and controls (P>0.05), although Group III hookworm patients and hookworm patients with their associating parasites had lower numbers of CD3 and CD4 cells.

Leucocyte Migration Inhibition Test (LMIT)

The LMIT percentages were lower in hookworm patient subgroups when compared to controls (P<0.05). Values for all hookworm patients put together was 45?9 (24-69%). These values are significantly lower than values for controls 56?6 (48-69%) (P<0.05).

Discussion

The results indicate that in all the patient subgroups, the relative percentages of total T-cells (CD3) and T-helper cells (CD4) were significantly decreased (P<0.05) while percentages of CD8 positive or T-suppressor cells were not significantly different

from controls. The CD4/CD8 ratio however insignificantly was decreased in patients when compared to controls; suggesting an enhanced T-suppressor effect in hookworm patients. Absolute numbers of the CD3 cells though increased were not significantly increased when compared to controls and this is probably due to the higher total white blood cell counts observed in patients. Furthermore, it should be noted that significant ($P < 0.05$) reductions in percentage values of CD3 and CD4 cells were observed in patients with polyparasitism (hookworm accompanied with other parasites) as opposed to those with hookworm alone, suggesting a further effect of immunosuppression by other parasites. Previous studies have shown that the severity of immunosuppression in hookworm infection based on CD3 and CD4 cell results was less from patients without anaemia, more in those with mild to moderate anaemia and most pronounced in patients with severe anaemia who also tended to have higher Stoll counts of hookworm ova. This is consistent with our earlier study using T-cell rosettes showing immunosuppression in hookworm infection where patients with superinfection and marked anaemia had the lowest T-cell percentages [5]. KRANTMAN *et al.* [14] reported that in children with iron-deficiency anaemia, secondary to hookworm infection of mild to moderate severity, the major finding was a subtle T-cell deficiency, whereas with more marked anaemia was associated higher immune deficiencies.

Although we did not use hookworm specific antigens in the leucocyte migration inhibition test, the result shows that the cellular immune deficiency is milder in patients with mild anaemia and worse in patients with severe hookworm anaemia and infection. Absolute CD8 numbers were also more increased in these LMI deficient, markedly anaemic patients than in those without anaemia and controls. These results suggest that there is an enhanced suppressor T-cell function limiting antigen induced lymphocyte proliferative responses as has also been observed in human schistosomiasis associated immunosuppression [15]. While an exact mechanism for the immunodepression of cell mediated immune responses is difficult to propose, the suggestion of PRITCHARD (16) that the worms release immunomodulatory factors in the form of parasite excretory-secretory products and enzymes which may initiate non-specific suppressor cell activity should be taken into account. PRITCHARD (1986) has also suggested that the metabolism of essential fatty acids (EPAS) and the consequent local eicosanoid release at the site of larval penetration causes local immunosuppression in the skin. Even though the patients in this study were at a stable state in which the parasites were evidently producing eggs in the gut, his speculation may be extended to the local site of attachment of these worms in the gut, with the consequent local immunosuppression at the point of attachment, enhancing non-expulsion of the worms.

The depression of cellular immune responses in this study may also be related intrinsically and functionally to release of interleukin -2(IL-2) and its receptor on T-cell surfaces or the binding of IL-2 to its receptor. JOSIMOVICH-ALASEIVIC [17], studying IL-2R levels in patients with parasitic diseases found only mildly elevated levels of IL-2R in hookworm patients as compared to the very significantly elevated levels in patients with infections like filariasis, malaria and strongyloidiasis. In a similar study GASTL et al (13), studying numerical and functional alterations of T-lymphocytes in human schistosomiasis, attributed the observed decrease in CD3 and CD4 cells in patients with schistosomiasis to a depressed capacity of mononuclear cells to respond to mitogens and antigens *in vitro* and an insufficient release of IL-2 into culture supernatant. It is known that CD4 cells, in the presence of accessory cells and repeated stimulation by antigen, produce IL-2 which binds to its receptor IL-2R to result in CD3 and CD4 cell proliferation (18). CD4 cell percentages were reduced in this study, especially in the subgroup with marked anaemia. Metabolic blockage by factors produced by hookworms or the effect of suppressor factors and effect of increased absolute CD8 cells on CD4 cells may be some of the factors that may affect the production of IL-2. Although we did not measure IL-2 levels in our study, the functional abnormalities of the T-cell subpopulations studied which revealed a significant reduction in percent leucocyte migratory response to PPD in hookworm patients in Nigeria [5] could lead to suggestions that suppressor mechanisms might include a deficiency of IL-2 production or decreased expression of IL-2R on responding cells.

The observed abnormal cellular immune numbers and functions in hookworm patients in Zaria indicate that excessive hookworm parasite load coupled with anaemia is associated with depression of cellular immune responses. While the immune depression observed does not reflect strongly on the numerical aspect, the functional depression of migratory inhibitory factor percentages suggests an intrinsic defect perhaps having to do with suppressor mechanisms, a failure of lymphocytes to proliferate appropriately or the presence of immature T-lymphocytes. There is a need for further research on the role of null cells and NK cells in hookworm infection to better aid in determining the role of cellular immunity in hookworm infections

References

1. Ogilvie, B.M. and Worms, M.J. (1976). Immunity to nematode parasites of man with special reference to Ascaris, Hookworms and Filariae. In "Immunology of Parasitic Infections". Cohen S. and Sadun, E.H. (eds). Blackwell Scientific Publ. London, pp. 380 - 407.
2. Collwell, D.A. and Westcott, R.B. (1973). Prolongation of egg production of *Niponstrongylus brasiliennsis* in mice concurrently Infected with *Nematospiroides dubius*. J. Parasit. *59*: 216.
3. Phillipp, M. (1984). Acetylocholinesterase secreted by intestinal nematodes: a reinterpretation of its putative role of "biochemical holdfast". Trans. R. Soc. Trop. Med. Hyg. *77*: 138-139.
4. Lapron, A., Dessaint, J.P., Hague, A., Awriault, C. and Joseph, M. (1983). Macrophages as effector cells in helminth infection. Trans. R. Soc. Trop. Med. Hyg. *77* (5):631-635.
5. Olatunde, B.O. and Onyemelukwe, G.C. (1994). Immunosuppression in Nigerians with hookworm infection. Afr. J. Med. *Sci*:221-225.
6. Faust, E.C., Beaver, P., Jung, R.C. (1964). In "Clinical Parasitology". Kingston, London.
7. Stoll, N.R. and Hausheer, V.C. (1926). Concerning two options in dilution egg counting: Small drop and displacement. American J. Hyg. *6*: 134-145.
8. Hsieh, H.C. (1971). Combining MTFC and Stoll dilution egg counting for species analysis of hookworm in man. Clin. J. Microb *4*: 25-39.
9. Dacies, J.V. and Lewis, S.W. (1975). Practical Haematology, 5th ed. Churchill Livingstone, Edinburgh and New York.
10. Gupta, S. and Good R.A. (1977). Subpopulations of

human lymphocytes I. Studies in immunodeficient patients. Clin. and Exp. Immunol. *30*: 222.

Rosenberg, S.A., David, J.R..(1970). Inhibition of leucocyte migration: An evaluation of this in vitro assay of delayed hypersensitivity in man to a soluble antigen. J. Immuno;l. *015*: 1447-1452.

Genta, R.N., Otesen, E.A., Neva, F.A., Walzer, P.D., Tanowitz, H.B., Wittner, M. (1983). Cellualr responses in human strongyloidiasis. Am. J. Trop. Med. Hyg. *32*:990-994.

Gaslt, G.A., Feldmeier, H., Doering, E., Korman, C., Defalla, A.A. and Peters, H.H. (1984). Numerical and functional alternations of lymphocytes in human schistosomiasis. Scand J. Immunol. *19*: 469-479.

Krantman, H.J., Young, S.R., Auk, B.J., O'Donell, C.M. Racholefsky, C.S. and Sholun, E.R. (1982). Immune function in pure iron-deficiency. American J. Dis. Child. 136(9): 840-844.

15. Butterworth, A.E., Taylor, D.W., Veith, M.C., Voelars, M.S., Dessaints, A., Sturrock, R.F. and Wells, E. (1982). Studies on the mechanisms of immunity in schistosomiasis. Immunologic Rev. *61*: 5-39.

16. Pritchard, D.I. (1986). Antigens of gastrointestinal nematodes Trans. R. Soc. Trop. Med. Hyg. *60(5)*: 728-734.

17. Josimovich-Alaseivic, O. Feldmeier, H., Zwingenbergen, K., Harms, G., Hahn, H., Scrisuphamint, M. and Diamanstein, T. (1988). Interleukin 2 receptor in patients with localised and systemic parasitic diseases. Clinical and Exp. Immuno. *72*:249-254.

18. Wardle, E.N. (1988). The bodys defence against parasites. Medicine Digest *14*(6): 3-7.