

The allergen specific B-cell response during immunotherapy

S. H. SPARHOLT, O. T. OLSEN* and C. SCHOU

ALK Research, Bøge Alle, Hørsholm, Denmark and *Gentofte Hospital, University of Copenhagen, Hellerup, Denmark

Summary

The paper examines the allergen specific B-cell response in peripheral blood from patients undergoing immunotherapy with house dust mite extract. The 12 patients were part of a double blind placebo controlled study, and they were treated with either *Dermatophagoides pteronyssinus* ($n=4$), *Dermatophagoides farinae* extract ($n=3$) (Alutard SQ, ALK, Denmark) or placebo ($n=5$). Blood was taken every fortnight on day seven after hyposensitization and tested for IgM, IgG, IgA and IgE antibody secreting cells (AbSC) to *D. pteronyssinus* and *D. farinae* allergens and for the total number of immunoglobulin secreting cells (IgSC). The data showed a maximum of approximately 120 *Der f* I+II specific AbSC/ 10^6 mononuclear cells (MNC). A comparison of specific AbSC to the major allergens of the two house dust mites demonstrated that there was no measurable species specificity in the B-cell response that could be correlated to immunotherapy with either of the two extracts. The specific IgM, IgG, and IgA response to *Der f* I+II was examined in the placebo (39 measurements) and the actively treated (56 measurements) groups, and the results demonstrated a significant rise in specific IgM and IgA AbSC following immunotherapy. The number of specific IgG AbSC did not change. There was a mean of less than one specific IgE AbSC/ 10^6 MNC, and no detectable change following the treatment. It is speculated that immunotherapy to inhalant allergens causes the induction of specific IgA AbSC. It would then be these partly differentiated plasma cells that are detected on their way to the bronchial or gut mucosa to exert their protective function mediated by allergen specific secretory IgA.

Clinical and Experimental Allergy, Vol. 22, pp. 648–653. Submitted 31 July 1991; revised 8 December 1991; accepted 11 December 1991.

Introduction

The clinical efficacy of specific immunotherapy as treatment for IgE mediated inhalant allergy is documented in several studies [1–6]. To explain the immunological mechanisms underlying this clinical improvement intensive research has concentrated upon the specific antibody response in serum with respect to class and subclass distribution (for review see [7]). However, it has not been possible to confirm any definite correlation [8].

Experiments have demonstrated that results obtained from measuring specific serum antibodies are different from the results obtained from specific antibody secreting cells (AbSC) with respect to amount and isotype distribution [9]. We have investigated the allergen specific B-cell response during immunotherapy. In experiments per-

formed with traditional vaccines it has been shown that circulating specific AbSC are found between day 5 and 9 following vaccination [10,11]. Using an analogous approach we hope to obtain knowledge about the allergen specific B-cell activation taking place as a response to the individual injection. Based on the principles of the so-called ELI-spot assay [12], we have developed assays to quantify B-cells secreting antibodies to *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* allergens. Using these assays it is possible to enumerate the individual cells secreting IgM, IgG, IgA, and IgE antibodies to the major allergens of the two aforementioned house dust mites, *Der p* I and II and *Der f* I and II. In this paper we present the results on the allergen specific B-cells in peripheral blood during immunotherapy. We have measured the number of circulating specific AbSC of the four isotypes from 12 patients during up dosing with either house dust mite extract or placebo.

Correspondence: Dr S. H. Sparholt, ALK Research, Bøge Alle 10-12, 2970 Hørsholm, Denmark.

Table 1. The general treatment protocol, which nine of the patients followed. Due to local reactions three of the patients received a slower dosing regimen

Injection no.	SQ units/ml	Volume (ml)	Dose (SQ units)
1	100	0.1	10
2	100	0.5	50
3	1000	0.1	100
4	1000	0.4	400
5	1000	0.7	700
6	10 000	0.1	1000
7	10 000	0.3	3000
8	10 000	0.5	5000
9	10 000	0.8	8000
10	100 000	0.1	10 000
11	100 000	0.2	20 000
12	100 000	0.4	40 000
13	100 000	0.6	60 000
14	100 000	0.8	80 000
15	100 000	1.0	100 000
Maintenance dose	100 000	1.0	100 000

Materials and methods

Patients

The 12 adult patients (2 males and 10 females) were part of a double-blind placebo controlled study (including a total of 30 patients) to describe the clinical effect of specific immunotherapy to house dust mite allergy. The patients were all asthmatics and selected on the basis of positive skin prick test (Soluprick, ALK, Denmark) and positive Magic Lite SQ specific IgE (ALK, Denmark) to *D. pteronyssinus* or *D. farinae*.

Treatment

The patients were receiving immunotherapy with either *D. pteronyssinus*, *D. farinae* extract (Alutard SQ, ALK, Denmark) or placebo, i.e. of histamine. The treatment consisted of weekly injections with increasing doses of extract for approximately 3 months followed by injections of the maintenance dosage every 6 weeks (Table 1).

Blood and isolation of mononuclear cells (MNC)

Blood was drawn on day seven after injection, where circulating specific B-cells were supposed to be found [10,11]. The first blood sample was taken before the start of immunotherapy, and the following samples were drawn approximately every fortnight during up dosing. The number of samples taken from the individual patients

varied from six to eleven. MNC were isolated from heparinized blood by Ficoll-Isopaque (Nycomed, Oslo, Norway) gradient centrifugation (400 g, 20°C, 30 min). The white blood cell layer was collected and washed four times in 10 ml RPMI-1640 medium (Gibco, Herlev, Denmark) (400 g, 20°C, 10 min). Finally the cells were resuspended in RPMI-1640 containing 10% foetal calf serum (FCS) (SeroMed, Roskilde, Denmark).

Isolation of allergens

D. pteronyssinus and *D. farinae* extracts were produced from full grown whole mite cultures (WMC) obtained from ALK, Denmark. The cultures were extracted in ammonium hydrogen carbonate buffer pH 7.6 (1:10 w/v, 5°C, 24 hr), followed by centrifugation (40 000 g, 4°C, 1 hr), dialysis (3 × 24 hr) and freeze-drying [13].

Excrement fractions were isolated from WMC. *Der p* I and II were obtained by acetone extraction and precipitation of the excrement fraction, followed by hydrophobic interaction chromatography as described previously [14,15].

Der f I and II were purified from WMC extract using hydrophobic interaction chromatography as applied for the purification of *Der p* I and II. Fractions containing *Der f* II were identified using an ELISA assay and a *Der f* II specific murine monoclonal antibody.

Counting of allergen specific AbSC

The number of allergen specific AbSC was measured by a modified ELI-spot assay as described in details previously [9]. In brief, MNC are incubated in allergen coated microtitre wells for 2½ hr in a humidified incubator (5% CO₂, 37°C). Following incubation and wash a peroxidase conjugated antibody to either human IgG, IgM, IgA or IgE were added and incubated for 60 min, 20°C. After another wash the bound antibodies were visualized by the addition of an agarose-substrate solution. After approximately 20 min blue spots appeared in the bottom of the well, each representing a specific AbSC. The spots were counted in a low magnification microscope.

In each blood sample the number of circulating IgM, IgG, IgA, and IgE AbSC to the two major allergens of *D. pteronyssinus* (*Der p* I and II) and *D. farinae* (*Der f* I and II) was measured. Furthermore the number of AbSC to the WMC extract from the two house dust mites was enumerated. Each measurement was performed in duplicates.

Counting of immunoglobulin secreting cells (IgSC)

IgM-, IgG-, IgA-, and IgE-SC were measured using the principles of a capture ELISA as described previously [9].

Table 2. The relationship between the number of *Der f* I+II specific AbSC and the *Der p* I+II specific AbSC (*Df* I+II/*Dp* I+II) for each of the 12 patients. In the right column the type of treatment administered to the individual patient is shown; 'pl' indicates treatment with placebo, 'pt' and 'f' indicates immunotherapy with *D. pteronyssinus* and *D. farinae* extracts, respectively

Patient	<i>Df</i> I+II/ <i>Dp</i> I+II	Treatment
a	1.3 ± 0.9	pl
b	3.0 ± 3.2	pl
c	1.2 ± 1.1	pl
d	1.6 ± 0.4	pl
e	2.0 ± 1.6	pl
f	1.1 ± 0.8	pt
g	1.6 ± 1.2	pt
h	1.7 ± 0.4	pt
i	1.7 ± 1.6	pt
j	1.0 ± 0.4	f
k	0.9 ± 0.5	f
l	2.5 ± 1.3	f

Each measurement was performed in four replicates. For the detection of IgE secreting cells (IgE-SC) a high affinity murine monoclonal antibody was applied.

Statistics

The data were computerized using the SPSS/Pc package. The Mann-Whitney U-test was used for statistical evaluation of isotype differences.

Results

Number of allergen specific AbSC

The maximal measured number of *Der f* I+II specific AbSC (all four isotypes) was approximately 120/10⁶ MNC. However, the allergen specific B-cell numbers were generally between 0 and 100/10⁶ MNC, with only a few measurements above 100 AbSC/10⁶ MNC with IgM and IgA as the dominating isotypes.

We have measured both the number of *D. pteronyssinus* and the number of *D. farinae* specific AbSC to see if there was any detectable difference in the species specific B-cell response that could be ascribed to immunotherapy with either of the two extracts. The number of *Der f* I+II specific AbSC has been compared with the number of *Der p* I+II specific AbSC of all the four isotypes for each of the patients (Table 2). The numbers indicate the mean for all the blood samples taken from each patient.

The results illustrate that all patients had the same or

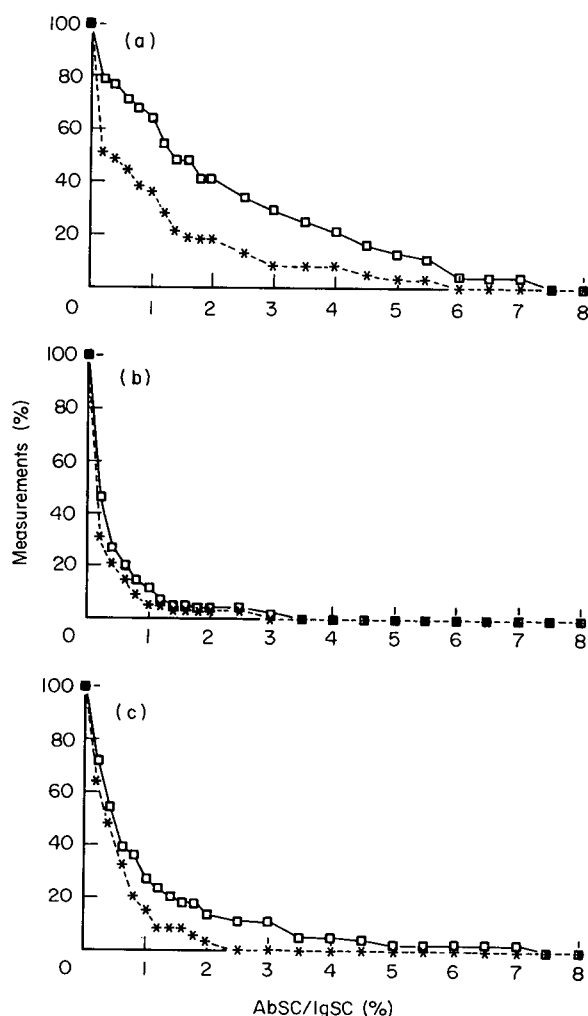


Fig. 1. The figure shows the number of (a) IgM, (b) IgG, and (c) IgA AbSC specific for *Der f* I+II. The lines represent the number of measurements (blood samples) where the percentage (AbSC/IgSC) of *Der f* I+II specific AbSC of the three isotypes is higher or equal to the number indicated on the x-axis. Measurements performed on blood samples taken before start of immunotherapy have been omitted. The solid line illustrates the results from the measurements on blood samples from patients who received immunotherapy with either *D. pteronyssinus* or *D. farinae* extract ($n=56$), and the dotted line indicates the measurements on blood samples obtained from the placebo treated patients ($n=39$).

more *Der f* I+II specific AbSC than *Der p* I+II specific AbSC. This included the five patients that received placebo treatment. Patient 'f, j' and 'k' exhibited approximately equal numbers of AbSC to the house dust mite species. The table also includes the treatment that the patients received, and as it is evident, there is no correlation between the number of specific AbSC to either *Der f* I+II or *Der p* I+II and the treatment with either of

the two extracts. Furthermore, all the placebo treated patients exhibited the highest B-cell response towards the *D. farinae* extract, which underlines the conclusion that the species specific B-cell response is not correlated to immunotherapy with either of the two extracts.

Analogous calculations have been performed to compare the number of *Der f* I+II specific AbSC and the *Der f* WMC extract specific AbSC, respectively. These results showed that for all patients, including the placebo treated patients, there was a stronger response towards the purified allergens than towards the WMC extract (data not shown). As a consequence of the aforementioned results the following presentation will concentrate on data obtained on *Der f* I+II specific AbSC.

The number of specific AbSC and IgSC vary considerably between individuals. In order to compare results obtained on allergen specific AbSC from the different patients the relationship (%) between the number of *Der f* I+II specific AbSC of each of the four isotypes and the corresponding number of IgSC from the same blood sample have been calculated. As no difference in the specific B-cell response to *D. pteronyssinus* and *D. farinae* extracts could be detected, the data from both these groups as a whole were compared with the data from the placebo treated group. The total number of measurements performed on blood samples from the placebo and the actively treated groups were 39 and 56, respectively.

Figure 1(a-c) shows the results obtained on *Der f* I+II specific IgM, IgG, and IgA AbSC. Figure 1(a) illustrates that there were more measurements exhibiting high percentages of IgM AbSC with specificity for *Der f* I+II in the group that received active immunotherapy than in the placebo treated group. The difference between the two groups was significant ($P=0.03$). In the actively treated group there were blood samples containing up to 7% IgM AbSC, whereas the placebo treated group exhibited a maximum of 5.5% *Der f* I+II specific AbSC. In general, the group that received immunotherapy with house dust mite extract has above 20% more measurements containing a given percentage of specific IgM AbSC than the placebo treated group.

The number of *Der f* I+II specific IgG AbSC is examined in Fig. 1(b). The results demonstrate that there was no detectable difference in the specific IgG B-cell response between the actively and placebo treated groups. The highest number observed was 3% for the actively treated group and 2.5% for the placebo treated group. In approximately 15% and 24% of the blood samples from the two groups, respectively, there was no *Der f* I+II specific IgG AbSC.

Figure 1(c) illustrates the number of *Der f* I+II specific IgA AbSC. As for the IgM AbSC there was a significant ($P=0.03$) increase in the number of IgA AbSC to the

major allergens in the group receiving the active treatment compared to the placebo treated group. In the actively treated group approximately 20% of the blood samples contained no IgA AbSC to *Der f* I+II and between 2 and 3% of the samples contained up to 7% IgA AbSC. In the placebo treated group the maximal amount was less than 2% IgA AbSC.

With regard to the IgE response, the highest number detected was four *Der f* I+II IgE AbSC/ 10^6 MNC, but the mean number was less than 1/ 10^6 MNC. No difference in the specific IgE response could be detected between the actively and the placebo treated groups.

Discussion

In this paper the allergen specific B-cell response to house dust mite allergens in peripheral blood from patients undergoing immunotherapy has been presented. The numbers of IgM, IgG, IgA, and IgE AbSC with specificity for the two major allergens and a WMC extract from *D. pteronyssinus* and *D. farinae* have been described.

Blood samples were taken from each of the 12 patients approximately every fortnight during up dosing. As indicated in Table 2, five of the patients had received placebo treatment, four patients immunotherapy with *D. pteronyssinus* extract and three patients with *D. farinae* extract. For each patient the results on AbSC to the purified allergens of the two house dust mite species were compared to describe any variance that could be caused by the difference in treatment. There was no correlation because all patients exhibited equal or higher numbers of *Der f* I+II AbSC than *Der p* I+II specific AbSC. This result corresponds with other experiments that demonstrate close physico-chemical and immuno-chemical similarities between allergens within the individual allergen groups [15]. It was therefore expected that immunotherapy with the one extract would activate B-cells, which for most of them would produce antibodies that cross-react to the other extract. However, this does not account for the generally stronger B-cell response to *D. farinae* allergens observed in both the actively and the placebo treated groups. An explanation could be a difference in the concentration of allergen bound to the solid phase. Quantitation of *Der f* I and *Der p* I using a commercially available kit [16] has demonstrated that there was 190 ng of *Der f* I bound to the microtitre well (solid phase), while there was only 140 ng of *Der p* I. This difference in bound allergen may mean that cells secreting antibodies of lower affinities might only be detected in the *Der f* I specific assay.

Analogous experiments demonstrated a higher number of specific AbSC to purified *Der f* I+II than to *Der f* WMC extract. The latter contains approximately 25

different components among which are the two major allergens. Although having the same molar concentrations and identical binding capacities only lower concentrations of these will bind to the solid phase compared to the purified allergens, because of the competition with the other components. Generally, lower concentrations of the individual antigens contained in the WMC extract are expected to bind, with the effect that fewer AbSC are detected.

In Fig. 1(a-c) the number of *Der f* I+II specific IgM, IgG and IgA AbSC were examined. The results demonstrated that there was a significant rise in specific IgM and IgA AbSC following immunotherapy with house dust mite extract compared to the placebo group.

The increase in specific IgA AbSC seems particularly interesting taking into consideration that house dust mite allergens are airborne inhalant allergens. As such they are introduced to the immune system through the mucosal surfaces in the airways or gut. From this point of view secretory IgA is likely to be involved. Mestecky and McGhee [17,18] have reviewed the role of IgA in the immune response of the gut and the respiratory tract, and they suggest that the B-cells that are detected in peripheral blood on day 5-9 upon vaccination [10,11] are partly differentiated plasma cells on their way to their effector tissue. A tempting hypothesis could be that immunotherapy causes the induction of allergen specific IgA AbSC, and that it is these cells that are on their way to the respiratory or gut mucosa and can be detected on day 7 following injection. Here they will exert their function mediated by protective secretory IgA. However, this theory has to be verified. First and foremost the clinical evaluation of the presented study has to be completed to actually elucidate whether a clinical improvement following the treatment can be correlated to the increase in specific IgA observed within the individual patient. This will be the topic of a subsequent paper. Furthermore, similar studies including more patients would be relevant to confirm the observed results. It is known that plasma IgA consists primarily of monomeric IgA and of the IgA1 subclass, while secretory IgA is dimeric and predominantly of the IgA2 subclass [19]. It would therefore be of interest to look at the antibodies from the individual cells to characterize the nature of the antibodies. In this way it will be possible to obtain an indication of the determination of the cells. An indication of the validity of the theory would also be demonstration of an increase in allergen specific secretory IgA in the respiratory tissues following immunotherapy.

The described rise in specific IgM AbSC following immunotherapy is possibly the result of a primary B-cell response.

The experiments showed no detectable difference in the

level of specific IgG AbSC between the two groups. Intensive research has concentrated on the allergen specific plasma IgG subclass distribution as a response to immunotherapy, but as mentioned earlier, it has not been possible to demonstrate any correlation. However, a rise in specific plasma IgG and IgE is described following the treatment [20], but a similar increase cannot be detected when looking upon neither allergen specific IgG AbSC nor IgE AbSC. This might be due to the overall concentration of specific antibodies of the two isotypes, which are probably too low to be detected in terms of a possible change in the number of specific AbSC. To describe a potential increase in specific IgE AbSC would require a volume of peripheral blood that is not realistic for the study.

As house dust mite allergens are inhalant airborne allergens and therefore supposed to involve the secretory immune response, it would be interesting to examine the allergen specific AbSC following immunotherapy with venom extracts. Is the treatment of stinging allergy and a positive clinical outcome succeeded by an increase in specific IgG AbSC to the venom allergens?

This study demonstrated a rise in allergen specific IgM and IgA AbSC following immunotherapy with house dust mite extract compared to a placebo group. There was no detectable change in the number of IgG and IgE AbSC with specificity for *Der f* I+II. It is speculated that by measuring on circulating specific AbSC instead of plasma antibodies we might have obtained knowledge about an effect of immunotherapy that could otherwise only have been obtained by looking on specific antibodies from the different mucosal tissues.

Acknowledgments

This study was supported by grants from the Danish Committee on Industrial Research Programme and the Danish Biotechnological Research and Development Programme. We thank Dr Vagn Andersen, Dr Ole Werdelin and Dr Torben Barington for valuable advice and Åse Brinch-Nielsen for technical assistance.

References

- 1 Mosbech H, Malling H-J, Biering I, Bøwadt H, Søborg M, Weeke B. Immunotherapy with yellow jacket venom. *Allergy* 1986; 41:95-103.
- 2 Nüchel Petersen B, Janniche H, Munch EP, Wihl J-Å, Bøwadt H, Ipsen H, Løwenstein H. Immunotherapy with partially purified and standardized tree pollen extracts, I. *Allergy* 1988; 43:353-62.
- 3 Sundin B, Lilja G, Graff-Lonnevig V, Hedlin G, Heilborn H, Norrlind K, Pegelow K-O, Løwenstein H. Immunotherapy with partially purified and standardized animal dander extracts, II. *J Allergy Clin Immunol* 1986; 77:478-87.

- 4 Wihl JÅ, Ipsen H, Munch EP, Janniche H, Løwenstein H. Immunotherapy with partially purified and standardized tree pollen extracts, II. *Allergy* 1988; 43:363–9.
- 5 Wahn U, Schweter C, Lind P, Løwenstein H. Prospective study on immunologic changes induced by two different *Dermatophagoides pteronyssinus* extracts prepared from whole mite culture and mite bodies. *J Allergy Clin Immunol* 1988; 82:360–70.
- 6 Østerballe O. Immunotherapy in hay fever with two major allergens 19, 25 and partially purified extract of timothy grass pollen. *Allergy* 1980; 35:473–89.
- 7 Djurup R. The subclass nature and clinical significance of the IgG antibody response in patients undergoing allergen specific immunotherapy. *Allergy* 1985; 40:469–86.
- 8 Østerballe O. Specific immunotherapy with purified grass pollen extracts. Copenhagen, Denmark, 1983.
- 9 Sparholt SH, Barington T, Schou C. Detection of B-lymphocytes secreting antibodies to *Dermatophagoides* antigens. *Clin Exp Allergy* 1991; 21:85–90.
- 10 Heilmann C, Pedersen FK. Quantitation of blood lymphocytes secreting antibodies to pneumococcal polysaccharides after *in vivo* antigenic stimulation. *Scand J Immunol* 1986; 23:189–94.
- 11 Barington T, Heilman C, Andersen V. Quantitation of antibody-secreting cells in the blood after vaccination with a *Haemophilus influenzae* type b conjugate vaccine. *Scand J Immunol* 1990; 31:515–22.
- 12 Czerkinsky CC, Nilsson L-Å, Nygren H, Ouchterlony Ö, Tarkowski A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J Immunol Methods* 1983; 65:109–21.
- 13 Lind P, Weeke B, Løwenstein H. A reference allergen preparation of house dust mite *D. pteronyssinus*, produced from whole mite culture—a part of the DAS 76* study. *Allergy* 1984; 39:259–74.
- 14 Lind P. Purification and partial characterization of two major allergens from the house dust mite *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* 1985; 76:753–61.
- 15 Lind P. Demonstration of close physicochemical similarity and partial immunochemical identity between the major allergen allergen Dp42, of the house dust mite, *D. pteronyssinus* and the corresponding antigens of *D. farinae* (Df6) and *D. microceras* (Dm6). *Int Arch Appl Immunol* 1986; 79:60–5.
- 16 Lind P. Enzyme-linked immunosorbent assay for determination of major excrement allergens of house dust mite species *D. pteronyssinus*, *D. farinae* and *D. microceras*. *Allergy* 1986; 41:442–51.
- 17 Mestecky J. The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J Clin Immunol* 1987; 7:265–76.
- 18 Mestecky J, McGhee J. Immunoglobulin A (IgA): molecular and cellular interaction involved in IgA biosynthesis and immune response. *Advances in Immunology* 1987; 40:153–245.
- 19 Kutteh WH, Koopman WJ, Conley MA, Egan ML, Mestecky J. Production of predominantly polymeric IgA by human peripheral blood lymphocytes stimulated *in vitro* with mitogens. *J Exp Med* 1980; 152:1424–9.
- 20 Creticos PS, Van Metre TE, Mardiney MR, Rosenberg GL, Norman PS, Adkinson NF. Dose response of IgE and IgG antibodies during ragweed immunotherapy. *J Allergy Clin Immunol* 1984; 73:94–104.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.