ENHANCED T CELL RESPONSES TO ANTIGENIC PEPTIDES
TARGETED TO B CELL SURFACE Ig, Ia, OR CLASS I MOLECULES

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Helper T cell activation by the globular protein antigens studied to date requires the uptake and intracellular processing of the antigen by an Ia-expressing APC releasing a peptide fragment containing the T cell antigenic determinant, which is transported to and held on the APC surface where the peptide is recognized by the specific T cell in conjunction with Ia (1–6). When APC-processing functions are blocked (2–6) or when Ia-containing planar membranes are substituted for APC (7), only peptide fragments or denatured proteins, not native proteins, activate T cells. While the requirement for the APC function in helper T cell activation is well documented, the molecular mechanisms underlying this phenomenon remain to be detailed. Of particular interest is the nature of the association of the antigenic peptide with Ia and with the TCR that leads to the activation of the T cell. Recent evidence indicates that peptides, but not native antigens, bind to Ia (8–10), and it has been suggested that T cells recognize a preformed peptide–Ia complex. Alternatively, T cells may facilitate the formation of such a complex (11), leading to their activation. In addition to Ia, other APC peptide-binding proteins have been implicated to play a role in antigen presentation (12, 13), possibly by transporting processed antigen to the cell surface and facilitating peptide interaction with Ia or the TCR.

In this report, we show that an antigenic peptide fragment of the soluble globular protein antigen, tobacco hornworm moth cytochrome c (THMc),1 residues 92–103 containing an NH$_2$-terminal cysteine (THMc92–103), is an extremely effective T cell antigen when covalently coupled to antibodies specific for Ig, Ia, or class I molecules expressed on surfaces of B cells used as APC. Thus, such peptide antibody conjugates were effectively presented by H-2$^k$ B cells to an I-E$^k$-restricted T cell hybrid, in an MHC-restricted fashion, requiring 100–1,000th of the peptide to reach maximal stimulation, as compared with THMc92–103 alone or THMc92–103 coupled to nonspecific Ig. Paraformaldehyde-fixed B cells, which

This work was supported by grants AI-18939, AI-12001, and AI-23717 from the National Institutes of Health. S. K. Pierce is a recipient of an American Cancer Society Faculty Research Award (FRA-279). Address correspondence to S. K. Pierce, Dept. of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, 2153 Sheridan Road, Evanston, IL 60208.

1 Abbreviations used in this paper: DTT, dithiothreitol; MBS, m-maleimidobenzoyl-N-hydroxysuccinimide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; S-SET, S-ethyl-thio; THMc, tobacco hornworm moth cytochrome c; THMc92–103, peptide representing the COOH terminus of THMc residues 92–103; THMc92–103, THMc92–103 with an additional NH$_2$-terminal cysteine residue.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/88/07/0171/10 $2.00

Volume 168 July 1988 171–180
are blocked in their processing functions, effectively present the peptide-antibody conjugates, even though, as previously demonstrated, B cells treated in this fashion fail to present native cytochrome c covalently coupled to the same Ig-, I-A\(^\alpha\), or K\(^\alpha\)-specific antibodies (14). Presentation is nearly as effective when B cells are incubated with the peptide antibody conjugate, and the unbound conjugate is removed before addition of T cells as when continuously present in T and B cell containing cultures, indicating that the stimulatory peptide antibody conjugates are associated with B cell surfaces. These results demonstrate that a small antigenic peptide covalently coupled to a larger antibody molecule can be effectively assembled into a stimulatory complex with Ia, even when the peptide antibody conjugates are initially bound to structures distinct from the Ia molecule recognized by the T cell.

Materials and Methods

**Animals.** CBA/J, C57BL/10J, and SJL/J female mice, 6–8 wk old, were purchased from the Jackson Laboratory, Bar Harbor, ME.

**Antibodies.** mAbs specific for I-A\(^\alpha\), a mouse IgG2\(\beta\)k antibody produced by the TIB93 cell line (15), for K\(^\alpha\), a mouse IgG2\(\alpha\)k antibody from the HB13 cell line (16), and for I-A\(^\*\), a mouse IgG2\(\beta\)k antibody derived from the HB4 cell lines (17), were each purified from culture supernatants as previously described (14). All cell lines are free of mycoplasma contamination, and antibody preparation is endotoxin free. Rabbit antibodies specific for F(ab\(^\prime\))\(_2\) fragments of mouse antibody (rabbit anti-Ig) were obtained from serum of rabbits immunized with F(ab\(^\prime\))\(_2\) fragments of mouse antibody purified by affinity chromatography on mouse-Ig Sepharose (14). Monovalent Fab fragments of rabbit anti-Ig were prepared by papain cleavage of affinity-purified antibodies as described (14). The monovalent fragments show a single silver stained band of 45 kD \(M_\text{r}\) by SDS-PAGE. Nonspecific rabbit Ig was obtained from nonimmune rabbit serum by chromatography on Protein A-Sepharose (14).

**Antigens.** THMc92-103 and THMcCys92-103 were synthesized by the solid phase method, according to the fluorenylmethoxycarbonyl (Fmoc)-t-butyl-strategy (18, 19) as previously described (14). Peptides were judged 98% pure by amino acid hydrolysis and HPLC analysis. THMcCys92-103 was covalently coupled to antibodies that had been modified with the heterobifunctional reagent \(m\)-maleimidobenzoyl-N-hydroxysuccinimide (MBS) (Pierce Chemical Co., Rockford, IL) as described (14, 20). THMcCys92-103, protected at its cysteine residue sulphydryl group with S-ethylthio (SSET), was iodinated with \(^{125}\)I by the chloramine T method (21), and \(^{125}\)I-peptide was added to unlabeled THMcCys92-103 in a ration of 1:20 to allow monitoring of the efficiency of the coupling procedure. THMcCys92-103 (10 mg/ml) in 25 mM PO\(_4\), pH 8.0, was reduced to remove the SSET-protecting group by addition of 40 mol excess of dithiothreitol (DTT) (Sigma Chemical Co., St. Louis, MO) and incubation for 24 h at 25°C. DTT was removed by gel filtration on a Sephadex B-25–150 column equilibrated in 0.1 M acetic acid. Peptide-containing fractions were pooled and lyophilized. Peptide was incubated with MBS-modified antibodies (3 mg/ml) (14) in 0.1 M PO\(_4\), pH 7.0, with 0.5 M NaCl for 24 h at 4°C, and the peptide-antibody conjugate was separated from free peptide by chromatography on a Sephadex G-75–40 column (50 cm \(\times\) 2 cm) equilibrated in PBS. Peptide antibody conjugates contain \(\sim\)1–2 peptides per antibody molecule and appear to be monomeric as judged by their behavior on the gel filtration column and by their mobility in nonreducing SDS-PAGE.

**Preparation of B Cells.** B cells used as APC were prepared from spleen cells of CBA/J mice by depletion of RBC by centrifugation on Ficoll-Hypaque gradients and T cells by treatment with mAbs directed toward Lyt-2, Thy-1, L3T4, and rabbit serum as a source of complement, as described (22). The resulting cell population was \(>95\%\) Ig positive and unresponsive to Con A (22). When indicated, B cells were fixed by treatment with 0.15% paraformaldehyde as detailed previously (6).

**Measurement of T Cell Activation.** The I-E\(^\alpha\)-restricted pigeon cytochrome c-specific T cell hybrid TPr9.1 secretes IL-2 in response to pigeon cytochrome c presented by H-2\(^\alpha\) APC (6)
and shows a heteroclitic response to THMc and THMc81-103 (23). TPc9.1 cells (5 x 10^4) were cocultured with B cells (2 x 10^5) and antigen in 0.2 ml cultures in 96-well microtiter plates in complete medium (22) containing 5% FCS, and the culture supernatants were removed 24 h later and assayed for the presence of IL-2 by the ability to maintain the growth of the IL-2-dependent cell line, CTLL, as previously described (24). CTLL viability was determined by the conversion of [3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co.) to a colored crystalline product absorbing at 570 nm when dissolved in isopropanol containing 0.04 N HCl, (25) or by the incorporation of [3H]thymidine (6).

Results

*THMcCys92-103-antibody Conjugates Are Effective T Cell Antigens.* The ability of the THMcCys92-103-antibody conjugates to activate I-E^k^-restricted THMc-specific TPc9.1 cells was evaluated when live B cells were used as APC. Because the unconjugated THMcCys92-103 peptide used to make the peptide antibody conjugates is antigenic and does not require processing, it was essential for this analysis to ensure that the peptide antibody conjugate contained no free peptide. As shown in Fig. 1, by SDS-PAGE analysis, the THMcCys92-103 normal rabbit Ig conjugate has been separated from the free peptide in the reaction mixture and contains undetectable free peptide. Similar results were obtained for each peptide antibody conjugate.

B cells were cocultured with irradiated TPc9.1 cells and graded concentrations of peptide or peptide-antibody conjugates, and the culture supernatants were tested 24 h later for the presence of IL-2 as a measure of T cell activation. The half maximal TPc9.1 response to THMcCys92-103-anti-Ig was achieved with ~1/1,000th the

![Figure 1. SDS-PAGE and autoradiography of ^125I-labeled THMcCys92-103-antibody conjugates. ^125I-labeled THMcCys92-103 was coupled to nonspecific rabbit Ig, and free peptide was separated from the peptide antibody conjugate by gel filtration as detailed in Materials and Methods. (Lanes 1–5) Peptide-normal rabbit Ig conjugate after gel filtration at 10, 20, and 40 μg, respectively. (Lanes 4 and 5) Peptide and rabbit antibody reaction mixture before gel filtration at 6 and 60 μg, respectively.](image)
Figure 2. THM-Cys92-103 covalently coupled to anti-Ig antibodies is a potent T cell antigen. TPr.1 cells (5 x 10^6) were cocultured with live B cells (2 x 10^5) as APC and varying concentrations of THM-Cys92-103 (A), THM-Cys92-103-rabbit anti-mouse Ig antibodies (O), THM-Cys92-103-nonspecific rabbit Ig (●), THM-Cys92-103 plus 20 μg/ml rabbit anti-mouse antibodies Ig (△), THM-Cys92-103 plus 20 μg/ml nonspecific rabbit Ig (□). Culture supernatants were removed at 24 h and tested for their IL-2 content by the MTT assay. The concentration of THM-Cys92-103 in the conjugates is shown on the ordinate.

Peptide required for unconjugated THM-Cys92-103, THM-Cys92-103 coupled to nonspecific rabbit Ig, THM-Cys92-103 plus rabbit anti-Ig, or THM-Cys92-103 plus nonspecific rabbit Ig (Fig. 2). Moreover, covalently coupling the THM-Cys92-103 peptide to nonspecific rabbit Ig does not affect its antigenicity, as compared with the free THM-Cys92-103, indicating that the peptide, when part of a larger protein structure, is as effective in activating the T cells as the free peptide. Like THM-Cys92-103, the TPr.1 response to the THM-Cys92-103-anti-Ig conjugate was MHC restricted in that no TPr.1 activation was observed when B cells were obtained from C57BL/10(H-2^b) mice, which fail to express an I-E molecule, or from SJL/J(H-2^a) mice (Table I). It should be noted that THM-Cys92-103 coupled to I-A^k-specific antibodies is somewhat more effective than unconjugated

Table I

Presentation of THM-Cys92-103-antibody conjugates is MHC Restricted

| Antigen               | CBA/J(H-2^b) | C57BL/10(H-2^b) | SJL/J(H-2^a) |
|-----------------------|--------------|-----------------|--------------|
| THM-Cys92-103         | 1.0          | >10.0           | >10.0        |
| THM-Cys92-103-anti-Ig | 0.001        | >0.7            | >0.7         |
| THM-Cys92-103-anti-I-A^k | 0.009     | >6.9            | >7.0         |
| THM-Cys92-103-anti-K^k | 0.007       | >5.8            | >7.0         |
| THM-Cys92-103-anti-I-A^k | 0.1        | >6.1            | >6.1         |
| THM-Cys92-103-NSIg    | 1.0          | >3.0            | >3.0         |

TPr.1 cells (5 x 10^6) were cultured with splenic B cells (2 x 10^5) purified from CBA/J(H-2^b), C57BL/10(H-2^b), or SJL/J(H-2^a) mouse spleens, and varying concentrations of THM-Cys92-103, THM-Cys92-103-anti-Ig, THM-Cys92-103-anti-I-A^k, THM-Cys92-103-anti-K^k, THM-Cys92-103-anti-I-A^k, or THM-Cys92-103-normal rabbit serum Ig. Culture supernatants were removed 24 h later and assayed for their IL-2 content by the MTT assay. The results are expressed as the concentration of THM-Cys92-103 required to achieve the 50% maximal TPr.1 cell responses.
The increased antigenicity of the peptide is also observed when THM{Cys}92-103 is coupled to antibodies specific for I-\(\alpha\) and K\(\beta\) (Fig. 3). Thus, the half maximal TP9.1 cell responses to THM{Cys}92-103-anti-I-\(\alpha\)\(^{\kappa}\) and to THM{Cys}92-103-anti-I-\(\alpha\)\(^{\kappa}\) conjugates were achieved at peptide concentrations 1/100-1/1,000th of that required for unconjugated THM{Cys}92-103, THM{Cys}92-103 coupled to antibodies specific for I-A\(^{\kappa}\), (Fig. 3) or to THM{Cys}92-103 plus either I-\(\alpha\)\(^{\kappa}\) or K\(\beta\) antibodies (Fig. 4). The TP9.1 response to THM{Cys}92-103 coupled to I-\(\alpha\)\(^{\kappa}\) and K\(\beta\)-specific antibodies is MHC restricted as B cells from C57BL/10J(H-2\(^{b}\)) and SJL/J(H-2\(^{d}\)) mice fail to present the conjugates (Table 1). The SJL/J(H-2\(^{d}\)) B cells also fail to present the THM{Cys}92-103-anti-I-A\(^{\kappa}\) conjugate, indicating that peptide bound to the APC surface, in the quantities achieved here, is not sufficient to activate the TP9.1 cells in the absence of the appropriate MHC molecules. Moreover, the effective presentation of THM{Cys}92-103 antibody conjugates to TP9.1 cells does not require the continued presence of the conjugate, in that B cells incubated with the THM{Cys}92-103-anti-Ig antibody conjugate for 2 h in complete medium, and exhaustively washed, activated TP9.1 cells to IL-2 secretion (data not shown).
Augmented Antigenicity of THMccys92–103-antibody Conjugates Does not Require Processing. THMccys92–103 covalently coupled to antibodies specific for Ig, (Fig. 5 and Table II), and for I-A\(^k\) and K\(^k\) (Table II), were nearly as effectively presented to TPc9.1 cells by paraformaldehyde-fixed B cells as by live cells, indicating that the peptide antibody complexes do not require processing. That the paraformaldehyde treatment did indeed block the processing function of the B cells is shown by the inability of fixed cells to present native Pc to TPc9.1 cells (Table II). In addition, fixed B cells were previously shown (14) to be incapable of presenting native cytochrome c covalently coupled to the same Ig, I-A\(^k\), and K\(^k\)-specific antibodies used here.

Fixed B cells were comparable with live B cells in their presentation of the peptide anti-Ig antibody conjugate, which in each case required 1,000-fold less peptide as

### Table II
Presentation of THMccys92–103-antibody Conjugates on Live and Paraformaldehyde-fixed Cells

| Antigen                        | [Antigen] required for 50% maximal T cell response when presented by: |
|-------------------------------|------------------------------------------------------------------------|
|                               | Live APC | Fixed APC |
| THMccys92–103                 | 1.1       | 4.0       |
| THMccys92–103-anti-Ig         | 0.002   | 0.004     |
| THMccys92–103-anti-I-A\(^k\)  | 0.001   | 0.01      |
| THMccys92–103-anti-K\(^k\)    | 0.003   | 0.02      |
| THMccys92–103-anti-I-A\(^k^k\)| 0.1     | 0.1       |
| THMccys92–103-Fab-anti-Ig     | 0.01    | 0.01      |
| Pc                            | 3.0      | >10       |

B cells from CBA/J(H-2\(^b\)) mice were untreated or fixed with paraformaldehyde and 2 \(\times 10^5\) cells cultured with 5 \(\times 10^4\) TPc9.1 cells and graded concentrations of THMccys92–103, THMccys92–103 coupled to antibody specific for Ig, I-A\(^k\), K\(^k\), I-A\(^k^k\), or to monovalent Fab fragments of rabbit anti-mouse antibody (Fab-anti-Ig) or with native Pc. The culture supernatants were removed 24 h later, and their IL-2 content was determined by the MTT assay. The concentration of antigen, either THMccys92–103 or Pc, required to achieve half maximal T cell responses is given.
compared with THMcCys92-103 alone (Table II) or with Pc8l-104 alone (Fig. 5). Although fixed B cells present the peptide anti-I-A\(^k\) and peptide anti-K\(^k\) antibody conjugates in an enhanced fashion requiring 1/400th the concentration of peptide alone, the presentation is less effective than that by live B cells. Fixed cells require ~10-fold more peptide to achieve maximal T cell responses as compared with live B cells. At present, the basis of the decreased ability of fixed B cells to present the peptide anti-Ia and peptide anti-class I antibody conjugate is not understood.

THMc92-103 Coupled to Monovalent Antibodies Is a Less Effective Antigen as Compared With THMcCys92-103-divalent Antibody Conjugates. To determine the contribution of antibody valency to the effectiveness of peptide antibody conjugates, THMc92-103 was coupled to monovalent Fab fragments of rabbit anti-Ig and the TPc9.1 response compared with that of THMcCys92-103 alone (Table II). The presentation of peptide coupled to monovalent fragments was enhanced ~100-fold as compared with THMcCys92-103 alone, which is 10-fold less effective than peptide coupled to intact bivalent rabbit anti-Ig antibodies. This was the case whether live or paraformaldehyde-fixed B cells were used as APC (Table II), suggesting that the efficacy of the bivalent conjugates may not be due to a need to patch Ig on the B cell's surface but rather to the increased avidity of the conjugate for the B cell's surface afforded by bivalent binding.

Discussion

Although the general features of antigen processing and presentation that lead to activation of Ia-restricted helper T cells are well documented, the molecular mechanisms underlying this phenomenon remain to be detailed. Of particular interest is the mechanism by which processed antigens or exogenously added antigenic peptides are held on the APC surface and recognized by the specific T cell in conjunction with Ia. Babbitt et al., (8) and Buus et al. (9, 10) demonstrated that peptides, but not native antigens, bind to purified Ia in detergent solution with a \(K_d\) of ~2 \( \times \) 10\(^{-6}\) M and suggest that the formation of a peptide-Ia complex may be a necessary first step in the activation of helper T cells. Subsequent studies by Watts et al. (26) indicated that ~1% of purified Ia incorporated into planar membranes bound peptide, in a nearly irreversible fashion, and activated specific T cells. However, earlier studies (11) indicated that peptide did not associate with Ia in planar membranes in the absence of a specific T cell, but did so when such T cells were present, suggesting that the TCR played a role in assembling an Ia-peptide complex. Indeed, APC structures, distinct from Ia, have been demonstrated to bind peptide and to play a role in antigen processing and/or presentation (12, 13), possibly by transporting processed antigen to the APC surface and facilitating its interaction with Ia and the TCR.

In this report, we show that the T cell–antigenic peptide, THMcCys92-103, covalently coupled to antibodies specific for the B cell surface structures Ig, Ia, and Class I are effectively presented to specific T cells by B cells as APC, and that such presentation does not require internalization or processing of the peptide antibody conjugate. We have previously demonstrated that native cytochrome \(c\) covalently coupled to the same antibodies specific for either Ig, Ia, or class I were extremely effective antigens in vitro, activating cytochrome \(c\)-specific T cells in an MHC-restricted fashion when present in 1/1,000th the concentration required of cytochrome \(c\) alone,
cytochrome ε coupled to nonspecific Ig, or cytochrome ε plus unconjugated antibodies (14). However, presentation of such conjugates, like the native cytochrome ε, required processing, in that the conjugates are not presented by paraformaldehyde-fixed B cells. Taken together, these results and those presented in this report demonstrate that a small 12-amino acid peptide covalently coupled to a larger macromolecule such as antibody does not diminish its ability to be assembled into a stimulatory complex with Ia and the TCR, and that peptides bound initially to surface structures other than Ia are extremely effective T cell antigens. A similar observation, concerning the antigenicity of peptides that are part of large macromolecules, was made by Lorenz and Allen (27), who showed that the T cell recognition of a COOH-terminal antigenic determinant of the 340-kD protein fibrinogen does not require processing.

An important question that remains to be answered is the location of the stimulatory peptide on the B cell surface when it is recognized by the T cell. It would seem unlikely that the peptide antibody conjugate undergoes a significant degree of proteolysis on the B cell surface, allowing the dissociation of the peptide in quantities sufficient for T cell activation. If this were the case, one would anticipate that the native cytochrome ε antibody conjugates would be active, to some degree, in the absence of processing. Indeed, if active proteolysis occurs at the B cell surface, the peptide itself would be extremely sensitive to degradation and consequent loss of activity. It has been shown that removal of the COOH-terminal lysine residues destroys the peptide's antigenic activity, and peptides shorter than 10 amino acids are insufficient to activate the T cell. Therefore, any postulated proteolysis would require the removal of the peptide from the Ig with no degradation of the peptide itself. However, the peptide antibody conjugate may not be bound to the structure for which the antibody is specific at the time it is recognized by the T cell in conjunction with Ia. The ability to pulse the B cell with the peptide antibody conjugate indicates that conjugates bound to the B cell are sufficient for T cell activation. However, the peptide antibody conjugate may be transferred from its binding site to a site on Ia or the TCR before T cell activation. Further studies, using chemical crosslinking reagents, may allow an elucidation of the position of the peptide antibody conjugate on the B cell surface.

The observation presented here, that short antigenic peptides covalently coupled to antibodies are effective T cells antigens in vitro, may provide a means to augment peptide immunogenicity in vivo. We have previously shown that native cytochrome ε covalently coupled to antibody specific for mouse Ig, which is ~1,000-fold more effective as an antigen in vitro as compared with cytochrome ε coupled to nonspecific Ig (14), is also immunogenic in vivo when administered to mice intravenously in saline, conditions under which native cytochrome ε fails to induce an antibody response (28). Results of studies to assess the efficacy of the THMcys92-103 in vivo should be of interest in this regard.

Summary

The helper T cell recognition of soluble globular protein antigens requires that the proteins be processed by an APC, releasing a peptide that is transported to and held on the APC surface where it is recognized by the specific T cell in conjunction with Ia. When cellular processing functions are blocked, APC lose their ability to
present native antigens while retaining the capacity to activate T cells when provided with a cognate peptide fragment that contains the T cell antigenic determinant. In this report, we show that a peptide fragment of the soluble globular protein antigen tobacco hornworm moth cytochrome c, residues 92-103 containing an additional NH2-terminal cysteine residue (THM-Cys92-103), is effectively presented by B cells to an I-Ek-restricted, THM-C-specific T cell hybrid when covalently coupled to antibodies specific for B cell surface Ig, Ia (A\(^b\)), or class I (K\(^b\)). Maximal activation of the T cells to the THM-Cys92-103-antibody conjugates is achieved with 1/100-1/1,000th of the peptide required using unconjugated THM-Cys92-103 or THM-Cys92-103 coupled to nonspecific antibody. The T cell response to the peptide antibody conjugates is MHC restricted, but unlike native cytochrome c-antibody conjugates, THM-Cys92-103-antibody conjugates do not require processing and can be presented by paraformaldehyde-fixed B cells. The THM-Cys92-103-antibody conjugate are nearly as effective when incubated with B cells, and the unbound conjugates washed away before addition of T cells as when continuously present in culture with T cells and B cells, indicating that the active peptide antibody conjugate is associated at the B cell surface. The presentation of THM-Cys92-103 coupled to monovalent Fab fragments of rabbit anti-Ig antibodies is less effective than that of the peptide coupled to bivalent antibody when either live or fixed B cells are APC, indicating that the avidity for the APC surface afforded by bivalent binding may be important in the conjugate's antigenicity. The results presented here indicate that a T cell-antigenic peptide, covalently coupled to a larger antibody molecule, can be readily recognized by an Ia-restricted helper T cell in the absence of processing. Moreover, the ability of the peptide to bind to B cell surfaces greatly augments the peptide's antigenicity, even when the binding is to structures distinct from the Ia molecule required for T cell activation.

Received for publication 19 January 1988 and in revised form 22 March 1988.

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