Biased Distribution of Recombination Sites within S Regions upon Immunoglobulin Class Switch Recombination Induced by Transforming Growth Factor β and Lipopolysaccharide

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Summary

We have characterized extrachromosomal circular DNAs from adult mouse spleen cells that were induced to switch to immunoglobulin A (IgA) with bacterial lipopolysaccharide (LPS) and transforming growth factor $\beta$ (TGF-β), and identified breakpoints of S#$/$S'1,3, S#$/$Sot, S3,3/$Sot, and S$\gamma$/Sot recombinants. The S#$ recombination donor sites clustered in the 3' half of the S$#$ region, while the Sot recombination acceptor sites clustered in the 5' half of the Sot region. In addition, donor and acceptor sites of S$\gamma$ regions also clustered in the 3' and 5' parts, respectively. These site preferences are in sharp contrast to the dispersed distribution of S#$/$S3,1 breakpoints within both S#$ and S'yl regions upon IgG1 switch induced by LPS and interleukin 4. Our results support the hypotheses that TGF-β increases the frequency of switch recombination events to IgA and that the switch recombination to IgA often proceeds by successive recombination of S#$/$S$\gamma$ and S$\gamma$/Sot.

Each class of the Ig has a unique effector function that determines how the antigen bound by the V region is processed and destroyed. A single B lymphocyte and its progeny continue to express a fixed V region that has been created by VDJ recombination. By contrast, Ig class that is defined by the C region of Ig H chain changes during the course of differentiation of a single B cell. The phenomenon, called class switching, is accompanied by DNA rearrangement, which takes place between switch (S) regions located 5' of each C$\alpha$ gene, except for the C$\delta$ gene (1-4). S-S recombination deletes an intervening DNA segment between a VDJ gene and one of C$\alpha$ genes, which are arranged in a linear order of 5'-C$\mu$-C$\delta$-C$\gamma$3-C$\gamma$1-Cy2b-Cy2a-Cc-C$\alpha$-3' in the mouse genome (5), thus bringing the VDJ gene in close proximity of the C$\alpha$ gene to be expressed (6-11). Each S region is composed of a tandem array of repeating units that differ from those of other S regions, but nonetheless, bear resemblance (12, 13).

The deleted intervening DNA is excised as a circular DNA (14-16). Since looped-out circular DNAs do not replicate, the nucleotide sequences of the fused S regions in circular DNA should reveal accurate locations of primary S-S recombination sites. Although switch recombination sites can be also examined by structural analyses of the switched Ig genes on the chromosome, direct assignments of chromosomal breakpoints are disturbed by the subsequent switch recombinations or secondary deletion of repetitive S regions. Sequential switch recombination reactions can be studied by analyses of breakpoint structures of the deletion products excised at each step of class switching.

In addition, relative frequencies of each S sequence in circular DNA can also serve as a direct measure of S-S recombination events that are regulated by cytokines. In the presence of the mitogen LPS, mouse spleen cells can be induced to switch specifically to IgG1 and IgA with IL-4 (17-19) and TGF-β (20, 21), respectively. These two cytokines have contrasting effects on spleen cells. For example, proliferative responses of spleen cells in vitro to LPS are depressed by TGF-β but not by IL-4. The percentage of IgA isotype induced by TGF-β is 10-fold lower than that of IgG1 induced by IL-4.

In this study, we assigned switch recombination sites induced by LPS plus TGF-β and made comparison with those induced by LPS plus IL-4. S$\mu$, S$\gamma$, and S$\alpha$ recombination breakpoints in the TGF-β-induced class switch are clustered at one end of the S regions in contrast to dispersed distribution of S$\mu$ and S$\gamma$1 breakpoints previously identified in the IL-4-induced switch.
Materials and Methods

Cell Preparation. Spleen cells were obtained from five 6-wk-old female BALB/c mice and cultured in RPMI 1640 supplemented with 10% FCS and antibiotics in the presence of LPS (30 μg/ml; Sigma Chemical Co., St. Louis, MO) and human TGF-β-1 (1 ng/ml; recombinant, generous gift from Dr. M. Sporn, National Institutes of Health; and purified, R & D Systems, Inc., Minneapolis, MN) for 3 or 6 d. Frequencies of switched plasma blasts were determined by surface staining of the cells with a FITC-labeled goat anti-mouse IgA (α chain specific; Cappel Products, Cooper Biomedical, West Chester, PA).

Construction of Circular DNA Clone Library. Circular DNAs were prepared from cells induced for 3 d (10^9) and 6 d (1.8 × 10^9), and uninduced cells (1.6 × 10^9) as previously described (22) with some modifications. Covalently closed circular DNA banded in a CsCl-ethidium bromide gradient was gently withdrawn through a wide-bore needle (22 gauge) to minimize the shearing forces. They were digested with XbaI and ligated with calf intestinal alkaline phosphatase-treated XbaI arms of the λZapII phage vector. The recombinant DNA was packaged in vitro. Phage titers per microgram of vector DNA was 1-3 × 10^6 for the recombinants.

DNA Hybridization. Plaque hybridizations were performed according to the methods of Maniatis et al. (23). All DNA probes shown in Fig. 1 and Table 1 were used as purified inserts. Every probe-positive clone was confirmed by the duplicate membranes.

DNA Sequence Analysis. XbaI-digested circular DNA clones were recloned into a pHSG399 plasmid vector. Nucleotide sequences were determined by the dideoxy chain termination method (24) using the universal M13 primer M4(M), reverse primer RV(R), or appropriate specific primers synthesized based on available sequences as follows. MUSIGCD18 (8,504 bp Sy3): TG4, CTGAGTGTTGCTTGG; MUSIGHAN A (2,705 bp Sy3): TG12, TAATTTTTCTATATTCC; TG19, GACAGCTCTGGAAGG; TG22, AGGAGAGGTGAAATTA; TG23, GATTATGGAAACCTTAAG; TG25, GCTGAGCTCTGCGG; TG27, GGGGAAGGTTGAGCTTAAT. Genomic region corresponding to the data base used and the orientation of sequencing primer are given in Fig. 1.

Results

Circular DNA Clones from Spleenocytes Stimulated with LPS and TGF-β. When mouse spleen cells were cultured in vitro by culturing with LPS and TGF-β, the frequency of IgA-positive blast cells increased from <0.1% to 3.8% after a 6-d culture. We prepared a cleared lysate of cultured spleen cells that either had not been stimulated or were stimulated with LPS and TGF-β and cultured for 3 or 6 d. Circular DNAs were purified as described previously (22) and cleaved at XbaI sites that are frequently found within or close to all S regions to isolate S-S recombinants of every combination of S regions (Fig. 1). We used the λZapII phage vector (cloning capacity up to 10 kb), which should be able to carry all S-S recombinants efficiently, except for the 12-kb XbaI fragment of the Sγ1 region. We screened and classified circular DNA clones by plaque hybridization with probes for S regions and Jκ sequences (Table 1). Frequencies of the Jκ-positive clones served as a monitor of cell proliferation, since the excision products of the VκJκ rearrangement are presumably unable to replicate, and thus diluted in the course of mature B cell proliferation (15). While frequencies of the Jκ-positive clones were decreased to 1/60 after extensive cell proliferation stimulated with LPS and IL-4 (15), LPS and TGF-β stimulation decreased
Table 1. plaque hybridization of circular DNA clones

| Stimulation | No. of clones screened | 3'-Sμ⁺ | Sγ3⁺/μ/γ3 | Sγ1⁺/γ3/γ1 | Sγ2⁺/μ/γ2, γ3/γ2 | Sc⁺/μ/α, γ3/α, γ2/α, X/α | Jk⁺ |
|-------------|------------------------|--------|-----------|-------------|-------------------|-----------------|-----|
| None        | 1.6 x 10⁶              | 3      | ND        | ND          | ND                | 1 (0, 0, 0, 1)   | 918 |
|             | 8.1 x 10⁵              | 0      | 0         | 2 (0, 0)   | 0                 | 0               | 488 |
| LPS + TGFβ  | 1.6 x 10⁶              | 60     | 74 (41)   | 2 (1)      | 87 (8, 16)        | 7               | 7 (0, 1, 4, 2)  | 536 |
| (3-d)       | 4.2 x 10⁵              | 20     | 27 (13)   | 0          | 22 (4, 6)         | 1               | 0              | 112 |
| (6-d)       | 8.0 x 10⁶              | 44     | ND        | 95 (6, ND) | ND                | 4 (1, 1, 2, 0)  | ND  |

Probe-positive clones were scored. Probes used: 3'-Sμ, 1.0-kb HindIII-XbaI fragment of plgH701-C (34); Sγ3, 6.0-kb Xbal fragment subclosed from Ch-M1-lg-γ3-30 (12); Sγ1, 0.5-kb BamHI-PstI fragment of pSy1.1AB (15); Sγ2, 6.6-kb EcoRI fragment of plgγ2b-26 (12); Sc, 6.1-kb Xhol-KpnI fragment (5); Jk, 1.7-kb HindIII-Xbal fragment (35). Locations of S region probes are indicated in Fig. 1. Score in parenthesis gives the number of double-positive recombinant clones except for the Sc⁺ recombinants with sequences whose origin was not identified in the other S region (X/α). S region isotype pairs in parenthesis are shown in the order of donor/acceptor.

The Jk⁺ clone frequencies only to one half in growth inhibitory effects of TGF-β on lymphocytes.

Probes from each S region were chosen so that recombination events of that region would be detected in our phage libraries. For instance, since the 3' Sμ region is deleted by any S-S recombination event involving the Sμ region, 3'-Sμ⁺ positive clones should contain the initial S regions paired with the Sμ region, which would be detected by subsequent probes as double-positive clones. Most of the Sμ regions were paired with either Sγ3 or Sγ2.

Most of Sγ3⁺ clones were recombinants with the Sμ, Sγ2, or Sc⁺ region. The Sγ2⁺ clones, which include both Sγ2b⁺ and Sγ2a⁺ clones due to cross-hybridization of the probe, recombinated with the Sμ, Sγ3, or Sc⁺ region, and some of the Sγ2⁺ clones may represent the Sμ germline sequence due to the multiple XbaI sites within the Sγ2b region. All the Sc⁺ clones seemed to be derived from the germline Sc sequence, as none of them hybridized with other S probes. These Sc⁺ germline sequences may have been excised by switch recombination between the Sμ and its upstream S regions. In fact, every Sc⁺-positive clone appeared to be a switch recombinant, and their number was equal to that of the germline Sc clones. Since no S-S recombination can give rise to circular DNA containing the germline Sc sequence, its absence in this phage library offers evidence for the purity of our circular DNA preparation.

The frequencies of 3'-Sμ⁺, Sγ3⁺, Sγ2⁺, and Sc⁺-positive clones increased drastically by LPS and TGF-β stimulation. The relative frequencies of acceptor S regions in Sμ recombinant clones decreased in the order of Sγ3, Sγ2, and Sc⁺, although frequencies of Sγ1⁺ clones may be underestimated owing to the cloning bias described above. Frequencies of Sc⁺ recombinants with Sγ2 were higher than those with Sγ3, and frequencies of Sγ3/Sγ2 recombinants were also higher than those of Sμ/Sγ2 recombinants. These results show that S-S recombination between more proximal S region pairs takes place more frequently.

Recombinant S sequences in circular DNA clones. We arbitrarily picked seven Sμ/Sγ3, two Sμ/Sγ2, and nine Sc⁺ clones from the libraries of circular DNA that were prepared from spleen cells stimulated for 3 or 6 d by LPS and TGF-β. Insert sizes of the 18 recombinant clones ranged from 0.7 to 4.4 kb, which are far less than the maximum packaging capacity (10 kb), suggesting the absence of cloning bias (Table 2). To determine the breakpoints of S region sequences, these clones were further studied by restriction enzyme mapping and DNA sequencing with specific primers.

We determined the nucleotide sequences of the breakpoints in 14 clones, as shown in Fig. 2. Comparison of the sequences surrounding the breakpoints with sequences of published germline S regions and overlapping circular DNA clones allowed us to assign the breakpoints on the germline DNA as depicted in Fig. 1. Breakpoints of the other four clones were mapped on the germline DNA by comparing nucleotide sequence ladders, although the accurate sequences were not determined (Table 2, Fig. 1). The Sγ2b breakpoint of pCS44 was located in a particular Sγ2b subregion whose sequence is unknown. The Sμ breakpoints of pCS23 and pCS18 were also located in an unsequenced Sμ region. These breakpoint sequences were compared conventionally with the prevalent consensus S sequences. Clone pCS31 contained the Sc⁺ sequence that abruptly switched to an unknown sequence whose origin was not found in the data base sequences of S, Dμ, and Jμ sequences. The recombinated sequence is similar to the Sc⁺ repeat unit, suggesting that this clone might have been generated by secondary recombination within the Sc⁺ region.
Table 2. Circular DNA Clones Analyzed

| Clone   | Size | Switch recombination | Breakpoint sequence determined |
|---------|------|----------------------|-------------------------------|
| pCS23   | 3.5  | μ/γ3                 | yes                           |
| pCS25   | 2.6* | γ3/α                 | yes                           |
| pCS29   | 2.4  | μ/γ3                 | yes                           |
| pCS28   | 2.8  | μ/γ3                 | yes                           |
| pCS36   | 3.3  | μ/γ3                 | no                            |
| pCS27   | 2.7  | μ/γ3                 | no                            |
| pCS35   | 1.8  | μ/γ3                 | yes                           |
| pCS22   | 4.4  | μ/γ2b                | yes                           |
| pCS24   | 3.5  | μ/γ2b                | yes                           |
| pCS18   | 3.5  | μ/α                  | yes                           |
| pCS42   | 3.9  | γ3/α                 | no                            |
| pCS19   | 1.9  | γ3/α                 | yes                           |
| pCS44   | 2.3  | γ2b/α                | yes                           |
| pCS43   | 0.7  | γ2b/α                | yes                           |
| pCS41   | 1.2  | γ2b/α                | yes                           |
| pCS32   | 1.7  | γ2b/α                | yes                           |
| pCS20   | 1.3  | γ2b/α                | yes                           |
| pCS31   | 1.5  | α/α                  | yes                           |

* Fragment size is shortened due to an internal deletion of 0.8 kb in Sy3.

at the recombination site was none for three clones, one base for four clones, two bases for five clones, three bases for one clone, and five bases for one clone (Fig. 2). An additional nongeornline nucleotide was inserted at the breakpoints of two clones, pCS22 and pCS20. Thus, the switch recombinations are obviously different from a typical homologous recombination and the common pentanucleotides CTGGG are found in close proximity to all of these S breakpoints except for Sy2b (Fig. 2).

Nucleotide Sequences of Sy and Sc Regions. The boundaries of the S region have been tentatively defined by hybridization of restriction DNA fragments surrounding C genes with the Sy and other S region probes (5, 13). The Sy breakpoint of pCS35 mapped to the 3' end of the Sy region, namely, the boundary between the homogeneous repetitive sequences consisting of GAGCT and GGGGT, and dispersed repeats with divergence.

The Sc breakpoints of the two clones, pCS43 and pCS44, were mapped outside the “Sc region” defined by Nikaido et al. (15), but within the 1.4-kb HindIII fragment cross-hybridizable with the Sa probe (5). We completed the 1,145-bp germline sequence downstream of the XbaI site located upstream of the Sc region by combining the sequences of overlapping Sc+ clones (Fig. 3). The 1,145-bp sequence was aligned with the prevalent 80-bp repeat unit of the Sc region (25). The 80-bp repeat unit was less obvious in the 5' part of the Sc region (63% homology) than in the 3' part of the Sc region defined by MUSIGCD41 (79% homology). However, this 1,145-bp sequence was comprised mostly of the simple repetition of the five-base consensus sequence,
CTGRG (R representing purine base), with which both the 5' and 3' parts of the Sx region could be aligned with 77% homology. This five-base (CTGRG) repeat unit, which is synonymous with the GRGCT repeat, has strong homology downstream of the XbaI site 5' to the Sx region was constructed from the overlapping Sx+DNA cloning capacity (9-23 kb) of the replacement type vector for the Sx region were identified in IgA (20, 21).

It is also striking that nine Sx acceptor sites that were used after stimulation with LPS and TGF-β were clustered in the 5' half of the Sx region. All four additional Sx acceptor sites used under similar conditions also fell within the same part of the Sx region (14). These results are in a sharp contrast to dispersed distribution of Sx donor sites identified in circular DNA derived from LPS/IL-4-stimulated spleen cells as shown in Fig. 1 (15).

In contrast, acceptor Sy1 sites used during class switching induced by LPS and II-4 are almost evenly scattered within the Sy1 region (see Fig. 1; reference 15).

Discussion

We have characterized relative frequencies and structures of recombinant S region sequences in looped-out circular DNA that was generated in murine spleen cells stimulated with LPS and TGF-β. Our cloning strategy allowed us to clone efficiently all switch recombinants and germline S regions except for the Sy1 region. All recombinant S regions except for the Sx region were identified in LPS/TGF-β-stimulated spleen cells. Our results are in agreement with previous reports that TGF-β increases the switch recombination frequency to IgA (20, 21).

A previous study has shown that the ratio of the frequency of the Sx recombination to that of the Sy recombination was >0.5 when spleen cells were stimulated with LPS and TGF-β (14). However, our analysis indicates that this ratio was >0.1. The difference could be due in part to cloning bias using different vectors and restriction enzymes. For instance, the cloning capacity (9-23 kb) of the replacement type vector phage (ADhII) used by Matsuoka et al. (14) excluded the oversized recombinants of Sy1/Sy2b, Sy1/Sy2a, Sy2b/Sy2a, Sy1/Sx, and Sy2b/Sx when cloned into EcoRI sites. Differences in Sx-cloning efficiency between EcoRI/λDhII library (14) and the present XbaI/λZapII library may also be due to the appearance of mitochondrial DNA clones in the library because all six mitochondrial XbaI fragments (7.6, 5.1, 1.9, 0.9, 0.5, and 0.3 kb) were packageable into the λZapII vector, whereas one out of three EcoRI fragments...
It is also important to compare frequencies of circular switch recombinants between spleen cells unstimulated and stimulated with LPS plus TGF-β. A similar comparison between spleen cells stimulated with LPS alone and LPS plus TGF-β (14) may not have represented effects of TGF-β properly. Spleen cells stimulated with LPS alone proliferate extensively whereas those stimulated with LPS and TGF-β grow very poorly as indicated by the frequency of the Jκ-positive clones (Table 1). Drastic difference in proliferation rate of spleen cells tends to bias frequencies of circular DNAs that do not replicate.

Most of the Sκ regions in circular DNA were recombinants with Sκ regions, indicating that class switching to IgA proceeds by successive S-S recombination. Initial evidence for the successive S-S recombination was obtained by identification of fusion of three S regions in IgG- and IgA-producing myeloma and IgE-producing hybridoma cells (2, 13, 25, 26). Recent studies on looped-out circular DNA containing hybrid S regions also demonstrated the existence of various combinations of S regions other than the Sκ region, such as Sy1/Sy2b, Sy3/Sκa, Sy2b/Sκa, Sy1/Sκb, Sy1/Sμ/Sκ, and Sy3/Sμ/Sκ (14, 27).

In previous work studying circular DNA derived from LPS/TGF-β-stimulated spleen cells, the frequencies of Sμ/Sκa and Sκ/Sκa recombinants were roughly comparable (14). In contrast, the present study showed that the majority of Sκ-containing circular DNA were Sr/Sκ recombinants. Again, the difference could be due to different cloning strategies between the two groups. However, it may be reasonable to speculate that Sy/Sκa recombination may be more frequent than Sμ/Sκa recombination in the presence of LPS and TGF-β because frequencies of switch circular DNAs spanning the shorter distance between S regions seem to be higher than those spanning the longer distance (Sy2-Sκa vs. Sy3-Sκa and Sy3-Sy2 vs. Sμ-Sy2). In case of class switching to IgE induced by Nippostrongylus brasiliensis, successive S-S recombination appears to be predominant (27). Taken together, class switching to the Cμ-distal Cκ genes such as the Ce and Cκ genes may often proceed by successive S-S recombination via Sy regions.

The breakpoints of the donor S regions are clustered in the S subregions proximal to Cκ genes, whereas those of the acceptor S region are in the S subregions distal to Cκ genes. These distributions are different from those of the class switch recombination induced by LPS and IL-4, in which the breakpoints tend to disperse in the Sμ and Sy1 regions (15). This biased distribution of the breakpoints cannot be explained by secondary deletions in the looped-out switch circles because, if such deletions were significant, even distribution of the Sμ and Sy1 switch recombination sites in IL-4-induced spleen cells (14, 15) would not have been observed.

In principle, the switch sites obtained by extrachromosomal analysis should agree with the actual chromosomal switch sites. However, chromosomal breakpoints were often located outside the switch repetitive regions, possibly due to the frequent secondary deletion in long-term cultured myeloma cells, virus-transformed cell lines, and hybridomas (1, 14, 28-30). Since looped-out circular S regions are unlikely to replicate as suggested by progressive dilution of Vκ/Jκ rearrangement products (15), whereas rearranged chromosomes continue several cycles of replication that increases the chance of deletion, it is reasonable to expect that the chance of S region deletion is less in the looped-out circle than in the chromosome and that the breakpoint structures identified in the circular DNA closely resemble the original recombinants.

The sequential recombination of Sμ/Sy and Sy/Sκa should result in a cluster of donor and acceptor Sy sites at the 3' and 5' parts of Sy regions, respectively (Fig. 4 A). If we assume preferred recombination between proximal S subregions, we can expect that donor Sμ and acceptor Sκa sites cluster in the 3' and 5' parts of their S regions, respectively. It is hard to determine with molecular biological techniques which of the Sμ/Sy and Sy/Sκa recombination events precedes. This distinction may require biological studies to test whether IgG expression usually precedes IgA expression in a B cell progeny. If the Sμ/Sy or Sy/Sκa recombination precedes Sμ/Sκa to bring the Sr region in proximity of the Sμ region, it is difficult to explain the preferred usage of the 3' part of the Sμ region or the 5' part of the Sκ region (Fig. 4 B). A direct class switch recombination between Sμ and Sκa regions would not explain the presence of Sy-Sκa recombinants (Fig. 4 C). However, all of these recombinations may not be mutually exclusive but simply differ in relative frequencies. Our data suggest that the model shown in Fig. 4 A may be most frequent.

The subregional difference in the S region usage between IL-4 and TGF-β may be explained by difference of either (a) switch recombinase or (b) chromatin opening region. It may

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**Figure 4.** Models for Sμ-Sκa recombination. (A) Sequential switch recombination; (B) switch recombination preceded by preliminary recombination; (C) direct class switch recombination. Number in parenthesis indicates the order of recombination events: E, enhancer; f, 1 exon for germline transcript; S, switch region; C, constant region; VDJ, rearranged variable region.
be possible that different switch recombinases are induced by IL-4 and TGF-β, and the TGF-β-induced enzyme(s) may prefer donor and acceptor subregions of S regions. The difference of recombinases may be due to modification of the same protein. In spleen cells stimulated with LPS and IL-4, conformation of chromatin structure of the switch region may be extensively changed by the mitogen-stimulated replication in such a way that the entire S region is activated and targeted by the switch recombinase. However, the mitogen-activated replication is suppressed in the presence of TGF-β, and the switch region for the acceptor isotype can be opened weakly for the recombinase, primarily from the upstream region preceded by the class-specific sterile transcription (31). In fact, germline transcription of the Cα gene induced by TGF-β is much weaker than that of the Cy1 gene induced by IL-4 (32).

It remains to be proven whether excision products of switch recombination are exactly reciprocal to the chromosomal products. Nongermline base addition at the breakpoint found in an Sμ/Sγ1 recombinant clone (15) does not appear to be accidental because of de novo base insertion in two additional clones pCS20 (Sγ2b/Sα) and pCS22 (Sα/Sγ2b). The de novo base insertion at the breakpoint suggests circularization of linear intermediates that may have been occasionally modified by base deletion or addition. No homologous sequences longer than the common pentanucleotides, GAGCT and GGGGT, were found even in the vicinity of breakpoints as indicated before (13). Therefore, switch recombinants may be classified as nonhomologous recombination that requires the minimal homology of one to six nucleotides for the joining reaction (33). The common pentanucleotide repeats may be essential requirements for the end joining in class switch recombinations and the recognition of a putative recombinase(s), as proposed earlier (13).

We are grateful to Ms. M. Yamaoka and H. Kanaya for help in preparing this manuscript, and Ms. J. Kuno and M. Wakino for technical assistance.

This work was supported by Grants-in-Aid for Science Research from the Ministry of Education, Science and Culture of Japan.

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Received for publication 22 November 1991 and in revised form 10 February 1992.

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