CONCISE COMMUNICATIONS

Effect of Human Immunodeficiency Virus Type 1 on Intracellular Activation and Superoxide Production by Neutrophils

José F. Muñoz,[1] Siham Salmen,[1] Lisbeth R. Berrueta,[1] Maria P. Carlos,[3] José A. Cova,[1] José H. Donis,[2] Manuel R. Hernández,[1] and José V. Torres[3]

[1]Clinical Institute of Immunology and [2]Department of Cardiology, University of Los Andes, Mérida, Venezuela; [3] Department of Medical Microbiology and Immunology, School of Medicine, University of California, Davis

The immunopathogenesis of AIDS is associated with the development of opportunistic infections by intracellular pathogens that can invade and reproduce freely because of impaired cellular functions. Neutrophils from asymptomatic human immunodeficiency virus (HIV) type 1–infected persons and from symptomatic patients with AIDS were found to retain normal phagocytosis activity while producing significantly less superoxide than neutrophils from HIV-1–negative subjects, when stimulated through Fc receptors or protein kinase C. After priming with a synthetic HIV-1 envelope peptide and stimulation via the Fc receptor, the neutrophils from HIV-1–negative controls had suppressed superoxide production, reduced phosphorylation of two unidentified cellular proteins, and increased expression of a third phosphoprotein. These results suggest that HIV-1 can produce direct functional damage of neutrophils through binding of envelope components to the cell membrane.

It is not known whether human immunodeficiency virus (HIV) type 1 directly affects the functional capacity of neutrophils; however, impaired neutrophil function has been observed in asymptomatic HIV-1–infected persons [1]. In patients with AIDS, suppressed superoxide production has been reported as decreased, increased, or unchanged [2–4]. Decreased chemotactic responses, phagocytic function, and superoxide production noted in neutrophils of some HIV-infected persons may be due to specific immunity or the use of antiretroviral, cytotoxic, or antimicrobial agents. These functional impairments could be accomplished by direct damage to the cell during binding of the virus or by infection of polymorphonuclear leukocyte (PMNL) cell progenitors. Such neutrophilic defects may predispose the infected person, particularly children, to bacterial infections [5, 6].

To examine the functional status of neutrophils from HIV-1–infected patients, compared with uninfected controls, we measured phagocytosis and superoxide production in freshly purified activated neutrophils obtained from peripheral blood. We also assayed for superoxide production in control neutrophils (from uninfected persons) exposed to synthetic peptides representing defined regions of the HIV-1 envelope glycoprotein.

Materials and Methods

Subjects. We studied 47 HIV-positive persons who lived in the state of Mérida, Venezuela. HIV status was confirmed by both ELISA and Western blot. Disease classification was according to CDC criteria [7]. Of the 47 HIV-positive subjects, 25 were asymptomatic (2 women, 23 men) and 22 had AIDS (2 women, 20 men). Forty HIV-1–seronegative persons were included as controls (17 women, 23 men). The mean ages in years ($\pm$SE) of study subjects were as follows: 32 $\pm$ 1.7 (range, 18–54) for the HIV-positive group; 31 $\pm$ 1.8 (range, 21–49) for the AIDS patients; and 33 $\pm$ 1.6 (range, 18–59) for the controls. The control group comprised 17 women and 23 men. All HIV-infected men were homosexuals, and all HIV-infected women were heterosexuals. Among asymptomatic subjects who were HIV-positive, 6 had received medication (4, zidovudine; 1, didanosine; and 1, trimethoprim-sulfamethoxazole [TMP-SMZ]). Among the AIDS patients, 8 had been treated with zidovudine and 4 with TMP-SMZ.

Analysis of lymphocyte subsets. Three-color flow cytometry was performed by FACSort (Becton Dickinson, San Jose, CA), following the manufacturer's instructions. We mixed 20 μL of TriTEST (anti-CD4, anti-CD8, and anti-CD3) reagent (Becton Dickinson) and 50 μL of whole blood and incubated the solution at room temperature for 15 min. After addition of lysing solution, the cells were mixed and analyzed by flow cytometry.

Received 11 June 1998; revised 20 January 1999; electronically published 28 May 1999.

All subjects gave written informed consent. Blood was collected from study participants in full compliance with an established protocol approved by the Universidad de Los Andes ethical committee.

Grant support: Consejo Nacional de Investigaciones Científicas y Tecnológicas (S1-96000321); Consejo de Desarollo Científico, Humanístico y Tecnológico de la Universidad de los Andes (M-542-95); National Institutes of Health (AI-35521 to JV.T.).

Reprints or correspondence: Dr. José V. Torres, Dept. of Medical Microbiology and Immunology, School of Medicine, Tupper Hall Room 3134, University of California, Davis, CA 95616 (jvtorres@ucdavis.edu).

The Journal of Infectious Diseases 1999; 180:206–10

0022-1899/99/8001-0029$02.00

Table 1. Phagocytic function and superoxide production by neutrophils from human immunodeficiency virus (HIV)-negative controls, HIV-1–positive asymptomatic subjects, and AIDS patients and superoxide production of neutrophils from HIV-1–negative subjects after priming with various strains of HIV-1 envelope glycoprotein (gp120) peptides.

Subject group (*n*)	CD4 cells/mm^3 ± SE	CD8 cells/mm^3 ± SE	Phagocytosis (% ± SE)	NBT reduction (mUDO/min ± SD)		
				Immunobeads	PMA	No stimulus
HIV-negative (40)	932 ± 23	502 ± 16	40.2 ± 2.2	8.2 ± 2.5	13.4 ± 6.4	1.2 ± 0.9
HIV-positive (25)	581 ± 68	1078 ± 89	40.9 ± 3.0	5.2 ± 2.9[a]	11.8 ± 5.4	1.1 ± 1.1
AIDS (22)	78 ± 12	739 ± 33	35.0 ± 4.0	5.9 ± 3.7[b]	8.8 ± 7.6[c]	0.7 ± 0.9

	Reduction (mUDO/min ± SE) after exposure to HIV-1 gp120 peptides						
	RFV3	SF2V2	LAI	Subtype A V3	MNV5	No peptide	Control
HIV-negative (34)							
No stimulus	0.5 ± 0.6	0.4 ± 0.7	0.5 ± 0.7	0.5 ± 0.6	0.6 ± 0.7	0.4 ± 0.6	0.4 ± 0.6
Immunobeads	8.4 ± 3.9	6.7 ± 3.5[d]	9.2 ± 4.9	7.4 ± 4.4	7.4 ± 4.0	9.3 ± 3.7	8.3 ± 2.6
PMA	11.5 ± 4.4	11.9 ± 4.5	11.1 ± 5.3	11.0 ± 4.8	10.5 ± 4.1	12.5 ± 4.6	11.0 ± 4.0

NOTE. Data are mean, SD, and SE. NBT = nitroblue tetrazolium; mUDO/min = mean increase in optical density reading unit/minute.
[a] $P < .001$ vs. HIV-negative subjects.
[b] $P = .005$ vs. HIV-negative subjects.
[c] $P = .006$ vs. HIV-negative subjects.
[d] $P < .001$, significant suppression vs. SF2V2-primed unstimulated neutrophils from HIV-negative subjects.

Neutrophil separation and phagocytic function. Blood was collected into citrate tubes, and erythrocytes were removed by sedimentation with 6% (wt/vol) dextran (Pharmacia Chemicals, Piscataway, NJ) [8, 9]. Diluted blood was layered onto ficoll gradient (Sigma, St. Louis) and centrifuged. The PMNL layer was collected, washed, counted, and resuspended to 40×10^6 cells/mL in Tyrode's buffer (1.3 m*M* NaCl, 2.6 m*M* KCl, 11 m*M* $NaHC0_2$, and 25 m*M* dextrose with Ca^{++} and Mg^{++}). Phagocytic function was determined in triplicate as previously described, with only slight modification [10]. Percentage of phagocytosis was calculated as the number of phagocytic cells divided by total cells counted × 100.

Measurement of superoxide production after neutrophil stimulation. We performed the nitroblue tetrazolium (NBT) reduction assay to determine superoxide production, as described elsewhere [11]: 96-well flat-bottomed polystyrene plates were coated with immunobeads (Irvine Scientific, Santa Anna, CA) at a concentration of 200 μg/well and with phorbol 12-myristate 13-acetate (PMA; Sigma) at a concentration of 250 ng/well. Control wells contained cells alone. Neutrophils (10^6 cells) from each group of subjects were added to the wells. Each sample was tested in triplicate. After the addition of the NBT solution (Sigma) to all wells, the plates were read at an optical density (OD) of 494 nm by use of a Microwell System ELISA plate reader (Organon Teknika, Durham, NC). The results were reported as mean increase in OD reading units per minute.

HIV-1 envelope glycoprotein peptides. Peptides were prepared as described elsewhere [12]. The synthetic peptides representative of various strains of HIV-1 envelope glycoprotein (gp120) consisted of amino acids (aa) RF3V3 (aa, 313–340), SF2V2 (aa,159–194), LAI (aa, 421–443), HIV-1 subtype A V3 (aa, 266–280), and MNV5 (aa, 454–476). A control peptide was also synthesized.

Priming of neutrophils from HIV-seronegative subjects. Blood was collected and processed, as described above [8, 9], from 34 HIV-seronegative persons (mean age, 28 ± 1.2 years; range, 20–59). We added 10 μg of the HIV-1 gp120 peptide panel and control peptide to tubes containing 2 mL of the neutrophil suspension (5×10^6 cells/mL). Control tubes containing cells alone were included. After incubation at 5°C for 45 min, the NBT reduction assay was performed as described above [11].

Tyrosine phosphorylation activity and protein expression of neutrophils from HIV-negative subjects after priming with SF2V2 peptide. We determined tyrosine phosphorylation patterns by antiphosphotyrosine blotting and total nonphosphorylated protein expression by SDS-PAGE in cell lysates, as described elsewhere [13–15].

Statistical analysis. Parametric data were analyzed by analysis of variance and Student's *t* test. To establish the differences of the HIV peptides on neutrophil function, comparative analysis was done by use of the InStat package (Graphpad Software, San Diego). $P < .05$ was considered statistically significant.

Results

Phagocytic function and superoxide production. Table 1 shows phagocytic function and superoxide production in neutrophils from HIV-positive asymptomatic subjects, AIDS patients, and HIV-negative controls. No significant difference ($P = .3$) in neutrophilic phagocytic function or in mean spontaneous superoxide production of neutrophils with the absence of a stimulus (control) was observed among the three groups ($P = .1$). However, the mean superoxide production of neutrophils after stimulation with immunobeads was significantly different between HIV-positive asymptomatic persons ($P < .001$) and AIDS patients ($P = .005$), compared with HIV-negative persons. After stimulation with PMA, significant differences were only found between the mean superoxide production of neutrophils in AIDS patients and in HIV-negative subjects ($P = .006$).

Table 1 also shows the results of superoxide production by neutrophils following priming with peptides representing sequences of the HIV-1 envelope glycoprotein. Among the neutrophils stimulated with immunobeads, only those primed with

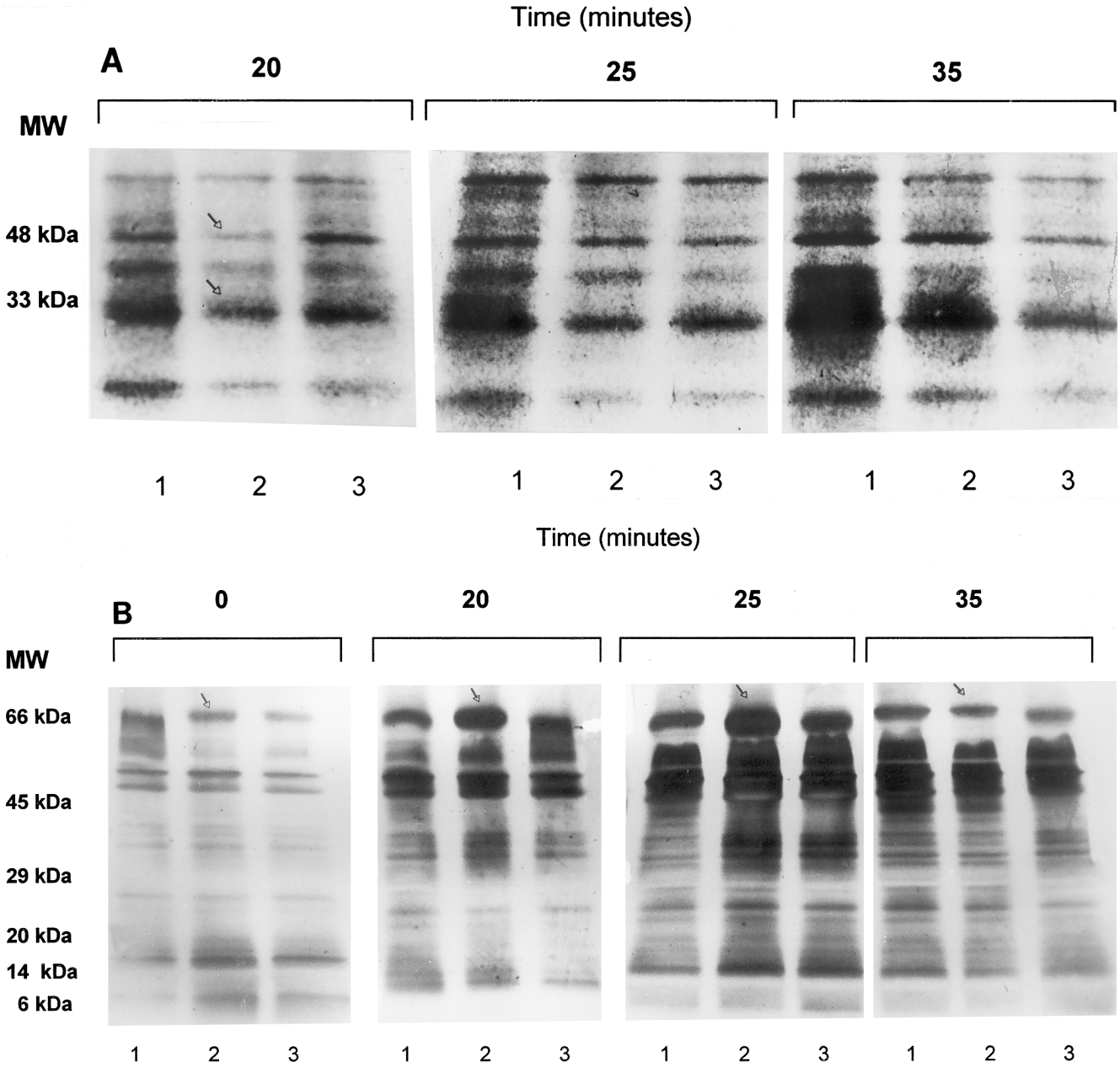

Figure 1. Immunoblot showing time course of tyrosine-phosphorylated protein with 4G10 monoclonal antibody (MAb) *(A)* and SDS-PAGE patterns of total nonphosphorylated protein *(B)* in cell lysates from neutrophils of HIV-1–negative subjects after incubation with SF2V2 peptide. Lanes 1–3 correspond to cell lysates of nonprimed, SF2V2 peptide–primed, and control peptide–primed neutrophils, respectively. *A,* Arrows indicate suppression of 2 phosphotyrosine proteins (48 and 33 kDa) in SF2V2 peptide–primed neutrophils. For each time point, cells were stimulated and then lysed. After immunoprecipitation, proteins were run on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and blotted with 4G10 MAb as described elsewhere [13]. *B,* Arrows show ~65-kDa protein overexpressed at 20 and 25 min. Whole cell lysates were resolved in 10% SDS-PAGE and silver stained.

peptide SF2V2 showed significant suppression in superoxide production, compared with unprimed controls ($P < .001$). No significant differences were observed between the primed and unprimed nonstimulated neutrophils. Similarly, no differences could be documented when both the primed and unprimed neutrophils were stimulated with PMA.

Tyrosine phosphorylation activity and protein expression of neutrophils from HIV-negative persons after priming with SF2V2

peptide. In figure 1*A,* the immunoblots show a steady increase in tyrosine phosphorylation with peptide SF2V2 over time. Twenty minutes after stimulus, the cell lysates primed with the SF2V2 peptide revealed a decrease in the expression of bands corresponding to phosphorylated cellular proteins of 48 and 33 kDa, compared with lysates from cells primed with the control peptide and nonprimed lysates. Figure 1*B* shows the steady increase in total nonphosphorylated proteins after priming with SF2V2 peptide. At 20–25 min, cell lysates from SF2V2-primed neutrophils revealed an increase in expression of a 65-kDa protein that was not seen in the control. At 35 min, this expression of the 65-kDa protein had returned to normal, which indicates that the suppression induced by SF2V2 peptide is relieved with time.

Discussion

Direct or indirect effects of HIV-1 infection on the functions of PMNLs have been implicated in the immunopathogenesis of AIDS [1]. However, the results have been contradictory, leaving largely unresolved the question of whether HIV-1 infection is accompanied by impairment of PMNL function [2–4]. These conflicting reports prompted us to investigate superoxide production by PMNLs after stimulation through 2 different pathways. We assessed the activity of the cell surface Fc receptor by using immunobeads and the activity of the protein kinase C intracellular signal transduction pathways by using PMA stimulation.

We found that superoxide production by neutrophils from HIV-1–positive asymptomatic persons or from AIDS patients was significantly suppressed when the Fc receptor was stimulated with immunobeads. In contrast, after PMA activation of the protein kinase C pathway, neutrophils from AIDS patients but not from HIV-1–positive asymptomatic persons had suppressed superoxide production, when compared with that in healthy control subjects. Although neutrophilic phagocytic function remained mostly intact, these results suggest some impairment in neutrophil function that might be associated with the secondary infections to which these HIV-1–infected persons are susceptible. The mechanism underlying this apparent suppression of superoxide production remains unknown, but it presumably represents an indirect effect of HIV-1 envelope or other viral proteins in so far as mature neutrophils are permissive to HIV-1 infection.

To investigate the possible role of HIV-1 envelope proteins on PMNL function, PMNLs from uninfected people were exposed to a series of synthetic peptides representing various regions of the HIV-1 envelope (table 1). These experiments revealed that only 1 peptide (from the V2 region of the envelope glycoprotein) suppressed superoxide production of the HIV-1 SF2 strain (B clade). Of interest, this suppression was seen when the Fc receptor was stimulated with immunobeads but not after PMA stimulation of the protein kinase C pathway. These results suggest that the HIV-1 envelope may exert an early toxic effect on PMNL function by down-regulating the Fc receptor–mediated activation pathway upstream of protein kinase C [2]. Blocking of the Fc receptor seems unlikely, since phagocytic function appeared to be intact. Alternatively, other cell receptors (e.g., CR3, interferon-γ, granulocyte–colony stimulating factor 2) or other secondary intracellular messenger pathways could be adversely affected by the HIV-1 envelope proteins. Consistent with this possible effect on signal transduction was our observation of the decreased levels of expression of some phosphotyrosine-containing proteins and increased expression of other proteins in lysates of control neutrophils exposed in vitro to the HIV-1 envelope peptide SF2V2.

These preliminary results suggest that HIV-1 proteins may interact with intracellular kinase/phosphatase pathways that are implicated in the superoxide production cascade. The documented increased expression of another protein might be a compensatory mechanism for the suppression of superoxide production. Identification of these proteins and their biochemical pathways will contribute to our understanding of the molecular mechanism underlying the apparent suppression of superoxide production by PMNLs from HIV-1–infected persons and may have relevance to the pathogenic effects of HIV. The identity of the proteins that are up-regulated and down-regulated as a result of neutrophil exposure to the SF2V2 peptide is being determined.

By directly inducing a suppressive effect on the ability of the neutrophil to produce superoxide, the capacity of this cell to destroy opportunistic pathogenic microorganisms could be heavily impaired. We are now testing whether the suppressive effect of this peptide can also be demonstrated in neutrophils from AIDS patients. Different V2 region analogs are being tested to determine if this assay can be developed into a pathogenicity marker for various HIV strains and molecular clones. Finally, blocking the PMNL surface region to which this domain binds could lead to a new therapeutic approach aimed at decreasing the incidence and severity of opportunistic infections in AIDS patients.

Acknowledgments

We thank José Ramirez for taking the gel photographs and Murray B. Gardner for reviewing the manuscript.

References

1. Valone FH, Payan DG, Abrams DI, Goetzl EJ. Defective polymorphonuclear leukocyte chemotaxis in homosexual men with persistent lymph node syndrome. J Infect Dis **1984**;150:267–71.
2. Gabrilovich DI, Kozhich AT, Suvorova ZK, et al. Influence of HIV antigens on functional activity of neutrophilic granulocytes. Scand J Immunol **1991**;33:549–52.
3. Chen TP, Roberts RL, Wu KG, Stiehm ER. Decreased superoxide anion and

hydrogen peroxide production by neutrophils and monocytes in human immunodeficiency virus–infected children and adults. Pediatr Res **1993**; 34:544–50.

4. Pinching AJ. The acquired immune deficiency syndrome. Clin Exp Immunol **1984**; 56:1–13.
5. Bondestam M, Hakansson L, Foucard T, Venge P. Defects in polymorphonuclear neutrophil function and susceptibility to infection in children. Scand J Clin Lab Invest **1986**; 46(7):685–94.
6. Simberkoff MS, Leaf HL. Bacterial infections in patients with HIV infection. In: Wormser GP, ed. AIDS and other manifestations of HIV infection. 2nd ed. New York: Raven Press, **1992**:269–75.
7. Centers for Disease Control and Prevention. Revision of the CDC surveillance case definition for acquired immunodeficiency syndrome. MWWR Morb Mortal Wkly Rep **1987**; 36(Suppl 1):1S–15S.
8. Southwick FS, Stossel TP. Phagocytosis. In: Rose NR, Friedman H, Fahey JL, eds. Manual of clinical laboratory immunology. 3rd ed. Washington, DC: American Society for Microbiology, **1986**:326–31.
9. Metcalf JA, Gallin JI, Nauseff WM, et al. Laboratory manual of neutrophil function. New York: Raven Press, **1986**:2–6.
10. Dunn PA, Tyrer HW. Quantitation of neutrophil phagocytosis using fluorescent latex beads: correlation of microscopy and flow cytometry. J Lab Clin Med **1981**; 98:374–81.
11. Virella G, Thompson T, Haskill-Strowd R. A new quantitative nitroblue tetrazolium reduction assay based on kinetic colorimetry. J Clin Lab Anal **1990**; 4:86–9.
12. Anderson DE, Malley A, Benjamini E, et al. Hypervariable epitope constructs as a means of accounting for epitope variability. Vaccine **1994**; 12:736–40.
13. Bennett PA, Finan PM, Doxon RJ, Kellie S. Tyrosine phosphatase antagonist–induced activation of neutrophil NADPH oxidase: a possible role for protein kinase C. Immunology **1995**; 85:304–10.
14. Al-Shami A, Gilbert C, Barabé F, et al. Preservation of the pattern of tyrosine phosphorylation in human neutrophil lysates. J Immunol Methods **1997**; 202:183–91.
15. Siegel JN. Antiphosphotyrosine blotting. In: Colligan JE, Kruisbeek AM, Margulies DH, et al., eds. Current protocols in immunology. Vol 3. New York: John Wiley and Sons, **1992**:11.3–11.34.