Effect of time-varying load on degree of bronchoconstriction in the dog

NORIHIRO SHINOZUKA,1 JEAN-PIERRE LAVOIE,3 JAMES G. MARTIN,1 AND JASON H. T. BATES1,2

1Meakins-Christie Laboratories, Royal Victoria Hospital, and 2Department of Biomedical Engineering, McGill University, Montreal H2X 2P2; and 3Faculté de Médecine Vétérinaire, Université du Montréal, St-Hyacinthe J2S 3B4 Quebec, Canada

Shinozuka, Norihiro, Jean-Pierre Lavoie, James G. Martin, and Jason H. T. Bates. Effect of time-varying load on degree of bronchoconstriction in the dog. J. Appl. Physiol. 85(4): 1464–1470, 1998.—It is well established that the degree of airway smooth muscle shortening produced by a given dose of bronchial agonist is greatly affected by lung volume. The airways are tethered by parenchymal attachments, the tension of which increases progressively with lung volume, thereby presenting a commensurately increasing hindrance to smooth muscle contraction. Earlier studies (P. F. Dillon, M. O. Aksoy, S. P. Driska, and R. A. Murphy, Science 211: 495–497, 1981) presented evidence that smooth muscle contraction initially involves rapidly cycling cross bridges, which then change to noncycling (latch) bridges. They also suggested that most of the muscle shortening occurs during the early rapid cross-bridge phase. This implies that smooth muscle subject to a given load early in contraction should shorten less than when it is subject to the same load later on. An in vitro study (W. Li and N. L. Stephens, Can. J. Physiol. Pharmacol. 72: 1458–1463, 1994) obtained support for this notion. To test this hypothesis in vivo, we measured the changes in lung impedance at 1 and 6 Hz produced in dogs by a bolus intravenous injection of methacholine when lung volume was increased for 10 s at different times after injection. We found that the changes in mechanics were greatly inhibited, whereas lung volume was elevated. However, when lung volume was returned to its initial level, the lung mechanics continued to change at a rate unaffected by the preceding volume change. We conclude that temporary mechanical inhibition of airway smooth muscle shortening in the normal dog in vivo merely delays an otherwise normal course of contraction.

airway smooth muscle contraction; latch bridge

AIRWAY SMOOTH MUSCLE (ASM) undergoes cyclic length changes during breathing, and there is evidence that this may significantly modulate the contractile behavior of the muscle when it is stimulated, either for mechanical reasons (11, 18) or via vagally mediated reflexes (6). It seems clear that, in normal individuals, stretching the ASM decreases the extent of its shortening, thereby reducing the degree of bronchoconstriction that would otherwise occur. Indeed, the bronchoconstriction that can be induced by agonist challenge in a normal lung can be almost entirely ablated by a few deep inspirations (9, 21). In asthmatic subjects, however, this effect is diminished or absent (9). Consequently, the effect of imposed length changes on the contractility of ASM is presently an area of considerable interest for the study of asthma and of airway responsiveness in general. Of considerable relevance in this regard is the well-established observation that the degree of ASM shortening produced by a given dose of bronchial agonist is greatly affected by lung volume (2, 3, 5, 8, 15–17, 21). This is almost certainly due in large part to the fact that the parenchymal attachments that tether an airway contribute significantly to the load against which the ASM must contract. The tension in these attachments increases progressively with increasing lung volume, thereby presenting a commensurately increasing hindrance to ASM contraction.

Dillon et al. (7) presented evidence that smooth muscle contraction initially involves rapidly cycling cross bridges, which then change to slowly cycling (latch) bridges some seconds after contraction begins. Furthermore, most of the muscle shortening occurs during the early, rapid, cross-bridge phase. This suggests that when ASM is subject to a given load early in contraction it should shorten less than when it is subject to the same load later on, because the early load would reduce the initial muscle contraction speed. Li and Stephens (12) obtained in vitro evidence to support this notion. The purpose of the present study was to test this hypothesis in vivo. Using previously developed methods (3), we continuously measured pulmonary mechanics in dogs during acute bronchoconstriction induced by iv methacholine (MCh). The time course of the load on the ASM was modulated by changing lung volume at different times after the beginning of contraction.

METHODS

We performed experiments on 11 mongrel dogs weighing 19–26 kg. The dogs were deeply anesthetized with a bolus of pentobarbital sodium (25–30 mg/kg iv) so as to abolish the corneal reflex. Anesthesia was maintained by additional bolus injections of 65 mg hourly. Paralysis was achieved with pancuronium bromide (0.5 mg iv) every 0.5 h. Each dog received a bolus injection of propranolol (2 mg/kg) and then 4.5–6 mg/kg propranolol every 0.5 h to mitigate functional antagonism caused by catecholamines released after repeated injections of MCh (see Reproducibility experiment). A rigid cannula (20 mm ID) was inserted into the trachea, and the chest was opened wide by midline sternotomy. The tracheal cannula was connected to a 50-ml piston oscillator. The cylinder of the oscillator had an occludable Y-piece sidearm, one branch of which was connected to a volume ventilator (model 618, Harvard Apparatus, South Natick, MA) and the other to a 2-liter plastic syringe. The expiratory line of the ventilator was connected to an adjustable water trap for applying a set level of positive end-expiratory pressure during experiments. Blood pressure in the femoral artery was monitored with a disposable blood pressure trans-
We drove the piston oscillator with a linear electric motor controlled by a computer via a digital-to-analog converter (DAC-02, Keithley, Taunton, MA). Piston position was recorded with a linear variable differential transformer (DE-C 1000, Shevaitz Engineering, Pennsauken, NJ). The position signal was calibrated in units of volume displacement to give a volume signal. Pressure at the tracheal opening (Ptr) was measured via a side tap between the piston and the tracheal cannula with a piezoresistive pressure transducer (Fujikura FPM-02PG; Servoflo, Lexington, MA). The measured signals (volume and Ptr) were low-pass filtered at 20 Hz with 6-pole Bessel filters and then sampled at 64 Hz with a 12-bit analog-to-digital converter (DT2801-A, Data Translation, Marlborough, MA). Acquisition of data and control of the linear motor were performed by using the LABDAT software package (RHT-InfoDat, Montreal, Quebec).

The purpose of our study was to investigate how bronchial responses are affected by changes in lung volume at various times during the development of the response to an applied agonist. This required that we repeat a standard measurement protocol a number of times in a given animal, each time varying the way in which lung volume was changed. Consequently, it was crucial that we achieve a repeatable degree of stimulation of the ASM from one run to the next. This is not something that can be taken for granted, as it is well known, for example, that the bronchial response to histamine exhibits marked tachyphylaxis in response to repeated application of histamine (1, 20). Tachyphylaxis in response to sequential challenges of MCh has been less well studied, although there is reason to believe that it may occur to some degree. Therefore, we first sought to establish the repeatability of the mechanical response to repeated intravenous (iv) MCh in dogs. Then we performed our main experimental protocol in another group of dogs. The complete experiment thus consisted of two parts: 1) a reproducibility experiment, and 2) a volume-change (ΔV) experiment.

Reproducibility experiment. This experiment consisted of a standard protocol that was repeated a number of times. The protocol began with a maneuver to standardize volume history. Three sighs to total lung capacity were achieved by briefly inflating the lungs to 3 kPa and then allowing complete expiration to an applied positive end-expiratory pressure level of 0.3 kPa. This was followed by 2 min of regular mechanical ventilation (20 breaths/min, 15 ml/kg tidal volume), after which the ventilator was turned off. The sidearm to the ventilator was then occluded to maintain the lung-piston oscillator system at a constant volume, during which time the piston was driven with an 80-s signal. The driving signal consisted of the superposition of 1- and 6-Hz sine waves with approximately equal power in flow at the two frequencies. The first and last 2 s of the signal were windowed by a cosine bell to avoid sharp on and off transients in the resulting Ptr signals. The peak-to-peak volume excursions of the piston were ~25 ml. At the beginning of the oscillations an iv bolus of 0.05 mg MCh was given through a central venous catheter via a femoral vein. The line was flushed with saline immediately after injection to ensure that the MCh was applied as sharply as possible. Immediately after the oscillations were finished, the sidearm to the mechanical ventilator was reopened, and regular ventilation was resumed. During collection of data, the heart was paced electrically at 2.5 Hz (5 V at 0.1-s pulse width applied directly to the right atrium) so that the frequency components of the cardio-genic oscillations in Ptr did not coincide with the frequencies (1 and 6 Hz) that resulted from the applied perturbations in volume. The pacing also presumably helped to ensure that cardiovascular function was maintained during the oscillations, as MCh slows the heart rate considerably and may even stop it completely for several seconds (although pacing would not have prevented any decrease in blood pressure that might have occurred due to peripheral vascular effects).

ΔV experiment. In each dog, we first measured the volume required to increase Ptr from 0.3 to 0.7 kPa by using a quasi-static volume titration procedure as follows. The procedure began with a maneuver to standardize volume history, as above, after which the ventilator was then turned off, a complete expiration was permitted, the connection to the ventilator was occluded, and the connection to a large syringe was opened. Volume increments of 100 ml were then applied to the lungs, with a pause of ~3 s between increments, until Ptr was >0.7 kPa. From these data we determined the ΔV that increased airway pressure from 0.3 to 0.7 kPa.

Next, we performed the main part of the protocol, consisting first of a control run, which was identical to that described above for the reproducibility experiments. This was followed by runs in which the lungs were briefly inflated during the development of the bronchoconstrictive response as follows. After a period of either 10 or 20 s from the beginning of the oscillations when the iv MCh bolus was given, the sidearm leading to the large syringe was opened, a ΔV of air was injected into the lungs, and then the sidearm was immediately closed. (The injection was done by a motor system over a period of ~2–3 s to avoid the development of very large pressures in the lung that would result from the air being injected too rapidly). After a further 10 s, an identical ΔV was withdrawn in the same manner. We performed this procedure in each animal, with volume increases applied at both 10–20 and 20–30 s after MCh injection; the order was varied among animals. We allowed 30 min of recovery between challenges.

Data analysis. The Ptr and volume displacement data we collected from the dogs during the 80-s postinjection periods contained information about the time evolution of pulmonary mechanical impedance at the two frequencies (1 and 6 Hz). The information contained in lung impedance at 6 Hz reflects, for the most part, the flow-resistive properties of the conducting airways. The information at 1 Hz reflects a combination of airway resistance and the mechanical properties of the lung tissues (3–5). The latter includes contributions from both tissue stiffness (i.e., the inverse of compliance) and tissue resistance (i.e., a reflection of the fact that stretching lung tissue dissipates energy as well as storing it). To extract this information, we had to subject the signals to a number of signal-processing procedures. We have described these procedures in detail in previous publications (3–5), so the analysis of our data was, for the most part, identical to that described by Bates et al. (3). First, we calculated the running mean of Ptr with a 1-s window to average out all of the 1- and 6-Hz signal sets by recursive least squares with a memory time constant of 0.5 s. This gave us 80-s signals of the form:

\[
Ptr(t) = ELV(t) + RLV(t) + K
\]

where t is time and K is a constant pressure, was fitted to both 1- and 6-Hz signal sets by recursive least squares with a memory time constant of 0.5 s. This gave us 80-s signals of the form:

\[
Ptr(t) = ELV(t) + RLV(t) + K
\]

where t is time and K is a constant pressure, was fitted to both 1- and 6-Hz signal sets by recursive least squares with a memory time constant of 0.5 s. This gave us 80-s signals of
l lung resistance (RL) and lung elastance (EL) at each frequency. For the remainder of the study, we will consider only RL at 6 Hz (RL6), because this is a useful measure of overall airway resistance and is somewhat easier to interpret than is RL at 1 Hz (RL1), which contains significant contributions from the lung tissues and also possibly from ventilation inhomogeneity after bronchoconstriction (3, 13). We will also consider only EL at 1 Hz (EL1) because, although inhomogeneities after bronchoconstriction can affect its value considerably, its magnitude is still easier to relate to the degree of bronchoconstriction than is EL at 6 Hz (EL6), which is significantly determined by gas inertance.

Statistical analysis. A one-tailed paired $t$-test was used to assess differences between lung mechanics parameters. Statistical significance was taken as $P < 0.05$.

RESULTS

Reproducibility experiment. Figure 1 shows the values of RL6 and EL1 obtained before each of the three sequential administrations of MCh in each of the five dogs studied for evidence of tachyphylaxis. Although there was individual variation among animals in prechallenge mechanics, there were no statistically significant differences between any pair of challenges. This shows that the animals began from the same baseline for each challenge. Figure 2 shows the maximum change in Pel ($\Delta$Pel) and the maximum values achieved by RL6 and EL1. Again, there were no significant differences in any of these quantities between challenges. Thus we demonstrated that our preparation did not exhibit any significant tachyphylaxis to repeated MCh injections.

$\Delta$V experiment. The $\Delta$V required to increase P$	ext{tr}$ from 0.3 to 0.7 kPa in the five dogs studied in the volume-loading experiment ranged from 450 to 900 ml. Figure 3 shows mean traces of RL6 and EL1 obtained under control conditions when lung volume was maintained constant throughout the entire 80-s measurement period (dashed line) together with the traces obtained when lung volume was increased by $\Delta$V between 10 and 20 s after MCh injection (solid lines). (Each individual data set was normalized so that the control data began at 0 and peaked at 100. The peaks in the curves in Fig. 3 have error bars, because the individual peaks occurred at different times.) Both RL6 and EL1 were markedly affected while lung volume was elevated; RL6 remained essentially constant near its baseline value during this time, and EL1 was raised slightly higher than the control curve. However, as soon as $\Delta$V was removed from the lungs at 20 s into the experiment, both RL6 and EL1 proceeded along trajectories very similar to the control curves, except that they were somewhat retarded. By the time the reactions peaked, at ~40 s, the two curves were almost superimposable (Fig. 3). There was no statistically significant difference between the peak values of the two curves.

The situation was similar with the curves obtained when $\Delta$V was applied between 20 and 30 s (Fig. 4). The control and volume-loading parameter curves were identical up to the point when $\Delta$V was injected (at 20 s). For the following 10 s, the progress of both RL6 and EL1 was again markedly altered, with RL6 being more severely reduced than shown in Fig. 3, while EL1 was increased, as shown in Fig. 3. However, as with the earlier volume injection, after the lungs were returned to their original volumes, both RL6 and EL1 proceeded as if they were retarded versions of their respective control curves. The peak in the volume-loaded curve for RL6 in Fig. 4 appears lower than the peak in the control curve, presumably because the control curve is waning at the time when the volume-loaded curves peaks. However, there was still no statistically significant difference between the peak values for both RL6 and EL1.

DISCUSSION

It is well known from in vitro studies that the mechanical load against which ASM must contract significantly influences the degree of shortening it can achieve. Numerous in vivo studies have confirmed the physiological significance of this phenomenon by showing that bronchial responsiveness is highly sensitive to lung inflation pressure or lung volume (1–3, 5, 8, 16, 17, 21). The load-opposing ASM contraction in vivo is greatly influenced by the tension in the parenchymal attachments that tether the airways, and this tension
is obviously determined in large part by inflation pressure. However, recent in vitro work by Li and Stephens (12) has suggested that the extent of ASM shortening may be significantly influenced not only by the magnitude of the external load but also by precisely when during the shortening process it is applied. The notion is that, early on during ASM contraction, relatively rapid shortening occurs by a process of rapid cross-bridge cycling. This gives way within a few seconds to a non-phosphorylation-dependent force-generation process that has been termed the latch-bridge state, during which tension is maintained but shortening rate is greatly reduced (22). The implication of this is that mechanically inhibiting ASM shortening early in the contraction process should lead to a greater reduction in total shortening than later application of the same inhibition. The goal of the present study was to test in vivo the physiological significance of this implication.

Before proceeding to address this goal, however, we first needed to establish a model of reproducible bronchoconstriction to repeated ASM stimulation, because our experimental protocol involves the use of each animal as its own control. The reproducibility experiments were thus designed to assess how well we could achieve a reproducible level of bronchoconstriction to repeated ASM challenge by intravenous administration of MCh. This agonist produces a number of effects in addition to bronchoconstriction, including reducing heart rate and inducing hypotension, which is a potent stimulus for catecholamine secretion by the adrenal gland and adrenergic nerves. We administered propranolol before MCh to inhibit the influences of catecholamines via receptors. This seems to have been effective, because we found no tachyphylaxis on average, although one dog clearly showed a progressively decreased response to repeated MCh (Fig. 2). It is presumably possible that tachyphylaxis in response to MCh could involve other mechanisms, such as changes in the mechanical properties of ASM or numbers of MCh receptors. Nevertheless, our results demonstrate overall that tachyphylaxis in response to repeated MCh administration was small in our preparation. Reproducibility of bronchoconstriction was also aided by the fact that we electrically paced the heart, which would have helped maintain blood pressure constant after MCh challenge and so would have reduced catecholamine secretion. Also, without cardiac pacing, the slowing of the heart rate would have altered the washout time of MCh from the lungs, presumably with a commensurate effect on the time course of bronchoconstriction. Finally, we used a rather small dose of MCh (1.9–2.6 µg/kg) to have a rapid recovery after each challenge. This ensured that the baseline values of the mechanical parameters were the same before each challenge (Fig. 1). It also meant that the degree of bronchoconstriction
achieved was not excessive, and thus the effects of regional inhomogeneities were minimized (3).

Our experimental approach of keeping the dogs apneic during the initial development of bronchoconstriction had the major advantage of allowing us to control the most important variable for our study, namely, lung inflation pressure. Conventional methods of studying airway responsiveness involve giving the agonist and then waiting until the response is fully developed before measuring respiratory mechanics. However, this does not control for the possibility that mean lung volume may change during the development period, for example by dynamic hyperinflation. This would not have been an acceptable risk to take for our present purposes, because airway responsiveness is so exquisitely sensitive to inflation pressure (3, 5). Of course, our approach gave us only a limited time window of 80 s from the time of MCh injection within which to observe the bronchoconstrictor response. However, we used a dose of MCh sufficiently small that the peak of the response was reached before 80 s; thus time was not a limiting factor in our experimental design.

We chose to monitor lung mechanics continuously during the 80-s experimental period in terms of two parameters (RL₆ and EL₁). We chose these two quantities because they can be followed continuously, with a high temporal resolution, by using our methods of digital filtering followed by recursive least squares model fitting. Also, we can interpret them in physiological terms on the basis of previous studies. Specifically, RL₆ has been shown to give a useful approximation to airway resistance during mild bronchoconstriction in dogs (4). In contrast, EL₁ is attributable to both the intrinsic stiffness of the tissues plus some contribution from regional ventilation inhomogeneity, the latter being a reflection of the degree of bronchoconstriction, which itself seems to be an inherently inhomogeneous process (3, 13). Of course, one might argue as to the precise extent to which these interpretations are true.
and whether, for example, inhomogeneities and changes in tissue resistance might affect RL6. Nevertheless, we contend that RL6 and EL1 together constitute a useful general measure of the degree of bronchoconstriction.

Another consideration for our study was to decide the appropriate time after the beginning of ASM contraction to apply our changes in lung volume. In our control experiments (those with no volume loading), the responses in both RL6 and EL1 began to be manifested at ~10 s after MCh injection, and they achieved their peaks at about 40 s after injection (Figs. 3 and 4). Therefore, by applying the changes in lung volume at 10–20 and 20–30 s, we effectively covered the times of major ASM shortening. Presumably, if any significant transitions from cross bridge to latch bridge occurred during the contractions, we would have selectively interfered with either one or the other in this way. Gerthoffer and Murphy (10) demonstrated a dissociation between force and shortening velocity in rabbit tracheal smooth muscle contraction induced by carbachol. Although active stress development reached a plateau at 2 min after the carbachol stimulation, shortening velocity peaked at 30 s; this result further supports the notion that a mechanical loading of the ASM between 0 and 30 s should have impeded the early rapid shortening due to cross bridges. Interestingly, Malmberg et al. (14) suggested that the lung inflations before airway challenge could have an important modulating effect on bronchial responsiveness, possibly through the release of bronchoactive mediators. Indeed, in preliminary experiments, we performed our 10-s injections of ΔV into the lungs very rapidly, generating large airway pressure swings during the injection and withdrawal phases. We noted that the animals’ responses to MCh after this maneuver seemed markedly ablated, so we employed a motorized system to make the ΔV somewhat more sedately over 1–2 s to reduce the magnitudes of the brief pressure fluctuations applied to the airway opening. It is thus possible that the application of large pressures to the airways has a modulating effect on their responsiveness, perhaps through the mechanism suggested by Malmberg et al. (14). This obviously warrants further investigation.

The key results of our study are shown in Figs. 3 and 4. In Fig. 3, the lung volume increase was applied at almost exactly the point (10 s) when the reaction in both RL6 and EL1 began. This essentially eliminated what would have been the first 10 s of ASM shortening (i.e., from 10 to 20 s after MCh injection), yet the curves obtained with the brief volume increase attained almost the same peak values as the control curves. Furthermore, the peaks in the volume increase curves for both RL6 and EL1 occurred ~10 s after the peaks of their respective control curves. After lung volume was returned to baseline, ASM shortening proceeded at a normal rate until the volume-loaded curve reached the control curve, and then the two curves remained close to each other as the ASM relaxed. This suggests that the effect of the 10-s volume increase was merely to retard the course of the ASM shortening by the same amount of time. Similar conclusions can be drawn from Fig. 4. Here the control and volume increase curves clearly matched each other until the lung volume was increased (at 20 s). Again, the volume increase effectively halted the shortening, and may even have reversed it somewhat. However, when lung volume was returned to baseline, shortening proceeded as normal, except that this time it was retarded by the ~20-s interval between the beginning of shortening and the removal of the ΔV. Our statistical analysis found no differences between the peak values of either RL6 or EL1 from the control experiments compared with the volume-loaded experiments shown in Fig. 4. This might seem somewhat at odds with the appearances of the curves themselves, which suggest that the volume-loaded peak for RL6 is lower than the control peak. The statistical result, therefore, probably reflects the scatter in the data. However, the time of the peak in the volume-loaded curve is later than that of the control curve, which itself is clearly decreasing by the time the volume-loaded curve peaks. Therefore, the value of the volume-loaded curve at its peak is even more similar to the control curve at the same time point than to the control curve at its peak. Because the drug delivery to the lungs was presumably the same under both control and volume-loaded conditions, this observation further supports the conclusion that the ΔV had only a transient effect on ASM contraction and did not affect the final degree of shortening.

The results shown in Figs. 3 and 4 do not support the hypothesis we set out to test, provided, of course, that the rapid cycling to latch-bridge transition in smooth muscle occurs within the time course of our experiment (i.e., within the first minute or so after the start of muscle simulation). That is, early brief interference with the course of ASM shortening does not seem to have significantly altered the subsequent rate of shortening, nor does it appear to have had a significant effect on total shortening. There is a suggestion that the volume-loaded curves did not achieve quite the same peak values as the control curves in Fig. 3 and especially in Fig. 4, but this was most likely simply because the response to MCh was waning by the time the volume-loaded curves peaked. Indeed, if the volume-loading maneuver had actually reduced the responsiveness of the ASM, we would have expected the volume-loaded curves to be below the control curves over the entire 80-s observation period instead of eventually “catching up” to the control curves, as seen in Figs. 3 and 4. These observations raise the question as to how important the time course of the transition from cross bridge to latch bridge is in determining airway responsiveness in vivo. Our results also suggest that deep breaths interfere with bronchoconstriction in normal subjects by a merely mechanical process in which transient loading of the ASM reverses shortening, as suggested by Sasaki and Hoppin (18). Regular tidal breathing would be expected to do the same, as demonstrated in rabbits by Shen et al. (19), although perhaps to a lesser degree, but it would have the additional feature of never allowing the ASM sufficient time to...
fully reestablish its unloaded shortening, because the risetimes of both $R_L$ and $E_L$ in Figs. 3 and 4 are on the order of 20–30 s. This is much longer than the period of a breath, so maximal shortening of ASM during an expiration could never be approached before the mechanical intervention of the next inspiration. This is not to say that pathological processes could not create abnormalities in the early rates of ASM shortening, as suggested by Stephens et al. (22) in the case of allergen-sensitized ASM. However, our results suggest that the time course of the mechanical load presented to the ASM is not an important mechanism, on its own, for explaining in vivo bronchial responsiveness.

This study was supported by the Medical Research Council of Canada, the J. T. Costello Memorial Research Fund, the Montreal Chest Hospital Research Institute, and the Canadian Network of Centres of Excellence in Respiratory Health (Inspiraplex). J. H. T. Bates was a Chercheur-Boursier of the Fonds de la Recherche en Santé du Québec.

Address for reprint requests: J. H. T. Bates, Meakins-Christie Laboratories, 3626 St. Urbain St., Montreal, Quebec, Canada H2X 2P2 (E-mail: jason@meakins.lan.mcgill.ca).

Received 19 September 1997; accepted in final form 1 January 1998.

REFERENCES


