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TOLERANCE TO SYMPATHOMIMETIC BRONCHODILATORS IN GUINEA-PIG ISOLATED LUNGS FOLLOWING CHRONIC ADMINISTRATION *in vivo*

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1 Pretreatment of the guinea-pig subcutaneously three times daily with either 5 μg/kg isoprenaline or adrenaline reduced the response of the isolated perfused histamine-constricted lung when challenged with either the same or a different sympathomimetic bronchodilator. The longer the animals were pretreated and the higher the dose of bronchodilator the greater was the degree of tolerance developed.

2 Tolerance was developed to aminophylline in the same preparation when the guinea-pig had been pretreated with aminophylline or isoprenaline. Cross-tolerance also developed to adrenaline when the guinea-pig had been pretreated with aminophylline.

3 Tolerance was still persistent in guinea-pigs one and two weeks after pretreatment three times daily with either isoprenaline or adrenaline (5 μg/kg s.c.) for seven days in the same preparation. Only after three weeks was the tolerance diminished.

4 These results suggest that asthmatic patients who use bronchodilators excessively may become refractory to the bronchodilator effect of these drugs. They also support the hypothesis that induced cross-resistance to endogenous sympathetic stimulation could lead to a deterioration of the asthmatic state in patients using the sympathomimetic bronchodilators and that this may explain the increase in asthma mortality rate.

5 A mechanism of tolerance to sympathomimetic bronchodilators is postulated.

Introduction

The asthma mortality rate in England and Wales increased during the period 1959-1966. This increase was real and not caused by environmental hazards or the wrong diagnosis of asthma death. It was related to the use of pressurized aerosols containing sympathomimetic bronchodilators which had become popular in the treatment of asthma since 1960 (Speizer, Doll & Heaf, 1968; Speizer, Doll, Heaf & Strang, 1968). Since 1968 further reports have been published which show a positive correlation between the use of pressurized aerosols containing sympathomimetic bronchodilators and an increase in the asthma mortality rate (Inman & Adelstein, 1969; Fraser, Speizer, Waters, Doll & Mann, 1971; Stolley, 1972).

Attempts have been made to find how these pressurized aerosols may cause the observed increase in asthma death. One explanation suggested by Conolly, Davies, Dollery & George (1971) is that excessive use of sympathomimetic amines by patients produces a state of drug-induced cross-resistance to endogenous sympathetic stimulation in the respiratory tract, which might lead to a deterioration in asthmatic state. The authors suggested that this might explain the rise in asthma mortality rate. The mortality experiments of Conolly *et al.* (1971) were verified by Bouhuys, Douglas & Lewis (1972).

In this paper a simple method has been used to investigate the development of tolerance and cross-tolerance to the bronchodilator effect of sympathomimetic amines in guinea-pigs. Recovery from this tolerance is also described.

Methods

The method was a modified version of that described by Sollman and von Oettingen (1928) and Tainter, Pedden & James (1934).

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A guinea-pig of either sex and weighing 400-500 g was killed by a blow on the head. The trachea and lungs were dissected free, and the trachea tied to a plastic cannula which was connected to a three-way tap through which drug solutions were injected. Slight scarifications were made along each lobe of the lungs with a fine needle. The trachea and lungs suspended from the cannula were surrounded by a heating jacket which was connected to a circulating water bath, so that the lungs were maintained at a temperature of 36-37° C. The lungs were perfused with Krebs solution of the following composition (g/litre distilled water):– NaCl 6.9, KCl 0.35, $CaCl_2$ 0.28, $MgCl_2$ 1.1, $NaHCO_3$ 2.1, NaH_2PO_4, $2H_2O$ 0.182, glucose 2. This solution was constantly aerated with a mixture of 95% O_2 and 5% CO_2 and passed through a coil immersed in a temperature-regulated water bath, so that the solution reached the cannula inserted into the lungs at 36-37° C. The Krebs solution then passed down the trachea through the bronchi, escaped from the alveoli through scratches on the surface of the lungs and was collected in a funnel. It then passed through a silicon rubber tube and entered a float recorder, which was a modified version of that described by Bülbring, Crema & Saxby (1958). When the Krebs solution reached a predetermined level in the measuring chamber of the flow recorder it emptied automatically. This caused a deflection to be recorded on smoked paper. Bronchoconstriction resulted in a reduced rate of flow and fewer signals on the smoked paper in a given time interval, whereas bronchodilation increased the rate of flow and frequency of signals.

Histamine was used as a bronchoconstrictor. The maximum response to histamine was obtained with a dose of 500 μg, and the response remained at this level for at least half an hour. Histamine was injected first, and the response recorded for 15 minutes. This was followed by injection of the bronchodilator drug, and the response to this drug recorded for 15 minutes. Doses of histamine and bronchodilator drugs were alternated in this way until the tissue failed to respond to the drugs. Tachyphylaxis to histamine developed in the isolated lungs. In order to compensate for this, the different doses of the bronchodilator drug given to the isolated lungs in all experiments were randomized.

Injection of solutions at room temperature into the isolated lungs was found to increase the flow rate of Krebs solution, so all injections of drugs were followed by injections of Krebs solution; thus a constant volume (1 ml) was given on each occasion. Control determinations, where the animals were not pretreated with bronchodilator drugs, were repeated at regular intervals throughout the experimental period. Each control curve shown in the results was the mean of a minimum of 15 determinations.

The animals were pretreated with bronchodilator drugs administered subcutaneously. Each dose of bronchodilator was given in 0.5 ml solution per kg body weight, three times daily for a varying number of days (1-14 days). Doses were calculated to body weight and chosen to be approximately the dose which an asthmatic patient might use in an attack. After the last injection, the animals were left overnight and killed the following morning. The number of animals used for each log dose-response curve was at least four, but usually more.

Drugs that were investigated were adrenaline acid tartrate, isoprenaline sulphate, salbutamol sulphate, terbutaline sulphate, aminophylline and histamine acid phosphate. The solutions of adrenaline, isoprenaline, salbutamol and terbutaline were stabilized with ascorbic acid 200 μg/ml.

To investigate the recovery from tolerance, the animals were killed 7, 14 and 21 days after completion of the course of pretreatment with isoprenaline or adrenaline for 3 or 7 days.

Increases in basal flow rates caused by increasing doses of bronchodilators were found to follow a log dose-response relationship. Tolerance to a bronchodilator was recognized as a downward displacement of the log dose-response curve of the isolated perfused histamine-constricted lungs of guinea-pigs which had been pretreated with either the same or a different bronchodilator when compared with the control curve of non-pretreated animals.

Statistical analysis was applied to the results obtained to test whether the log dose-response curves were altered. The significance of the vertical distance between the response mean of each curve was tested (Moore & Edwards, 1965). This parameter was selected in preference to the more conventional 'shift to the right' because in so many cases it proved impracticable to determine complete dose-response curves for each experiment.

Results

There was a downward displacement of the log dose-response curve of the isolated histamine-constricted lungs of the guinea-pig to adrenaline when the animals were pretreated for three days with adrenaline 5 μg/kg subcutaneously thrice daily ($P < 0.01$) (Figure 1).

The downward displacement of the curve was increased when the animals were pretreated for a longer period of time. Following pretreatment for

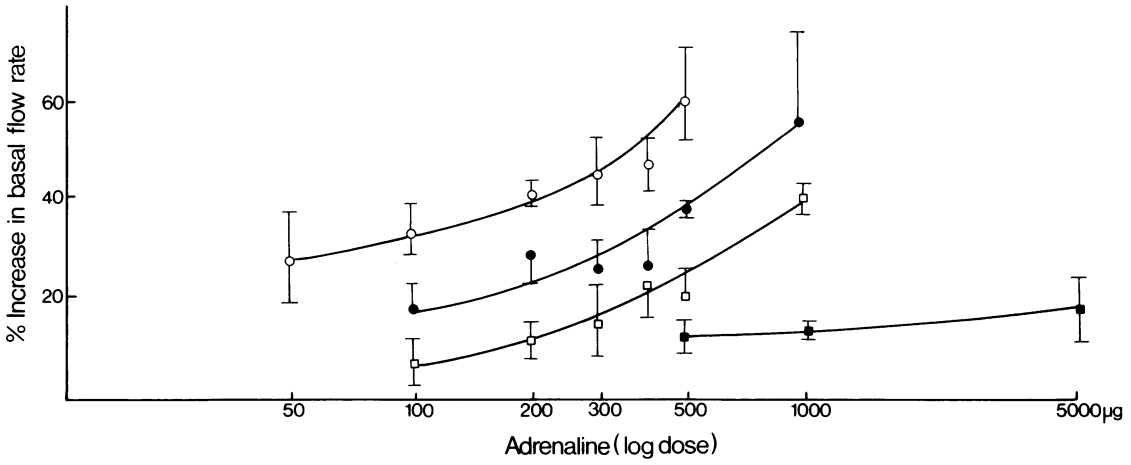

Figure 1 The effect of pretreatment with adrenaline (5 μg/kg) on the response of isolated perfused histamine-constricted guinea-pig lungs to adrenaline. (○) Control; (●) 3 days pretreatment; (□) 7 days pretreatment; (■) 14 days pretreatment. Vertical lines show s.e. mean.

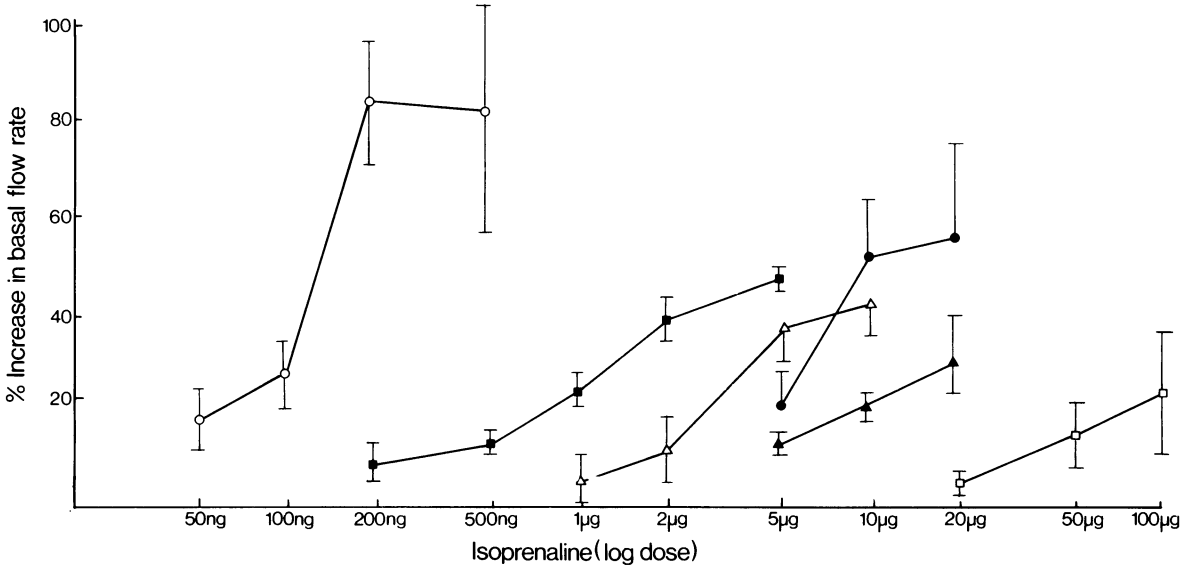

Figure 2 The effect of pretreatment with isoprenaline (5 μg/kg) or adrenaline (5 μg/kg) on the response of the isolated perfused histamine-constricted guinea-pig lung to isoprenaline. (○) Control; (■) 1 day pretreatment with isoprenaline; (△) 2 days pretreatment with isoprenaline; (●) 4 days pretreatment with isoprenaline; (□) 7 days pretreatment with isoprenaline; (▲) 4 days pretreatment with adrenaline. Vertical lines show s.e. mean.

two weeks, no significant response was obtained to doses of up to 5 mg.

When the animals were pretreated with a higher dose (10 μg/kg), the downward displacement of the log dose-response curve was increased.

Pretreatment of the animals with ascorbic acid 200 μg/ml in 0.9% w/v NaCl solution resulted in a response of the isolated histamine-constricted lungs to adrenaline which was not significantly different from that of non-pretreated animals.

A similar downward displacement of the log dose-response curves to isoprenaline was observed when the animals were pretreated with isoprenaline 5 μg/kg subcutaneously thrice daily for

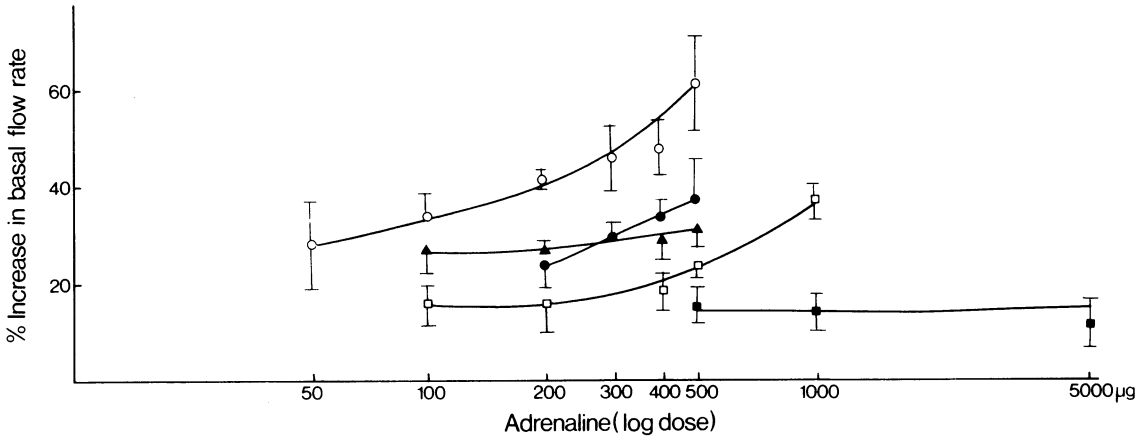

Figure 3 The effect of pretreatment with isoprenaline (5 μg/kg) on the response of isolated perfused histamine-constricted guinea-pig lungs to adrenaline. (○) Control; (●) 3 days pretreatment; (▲) 4 days pretreatment; (□) 7 days pretreatment; (■) 14 days pretreatment. Vertical lines show s.e. mean.

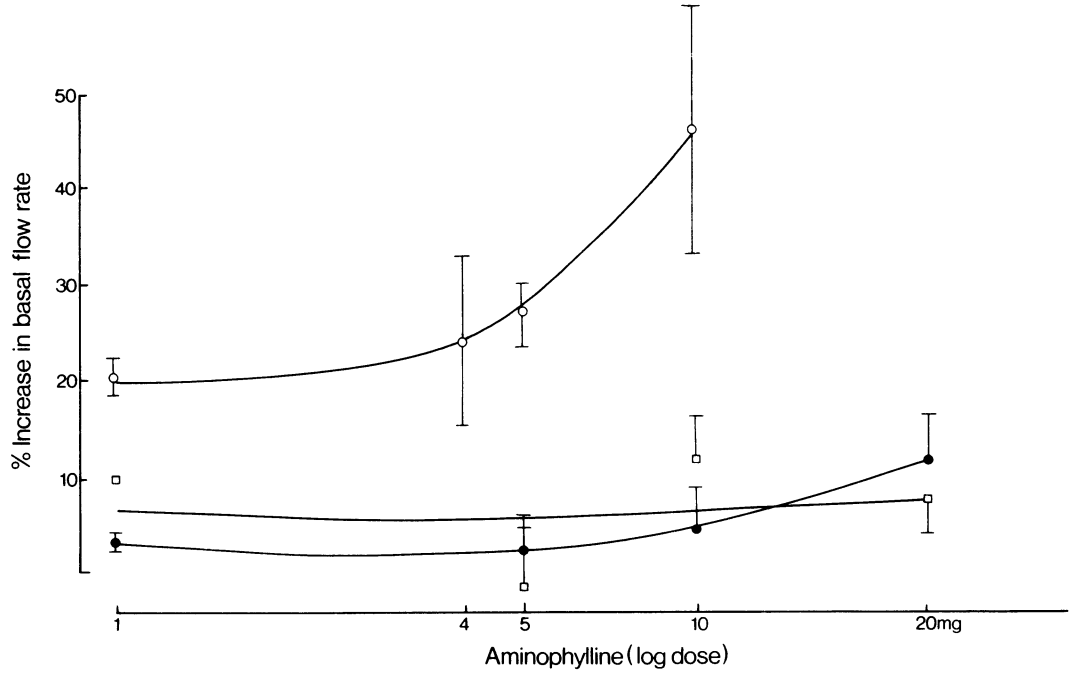

Figure 4 The effect of pretreatment with aminophylline (5 mg/kg) or isoprenaline (5 μg/kg) on the response of isolated perfused histamine-constricted guinea-pig lungs to aminophylline. (○) Control; (●) pretreated with aminophylline for 7 days; (□) pretreated with isoprenaline for 7 days. Vertical lines show s.e. mean.

one day ($P < 0.05$) (Figure 2). Increased pretreatment times produced further marked displacement of the curves to the right. The displacement of the curve was even more marked with an increased pretreatment dose (15 μg/kg). A similar effect was observed when the animals were pretreated with adrenaline and challenged with isoprenaline (Figure 2) or pretreated with iso-

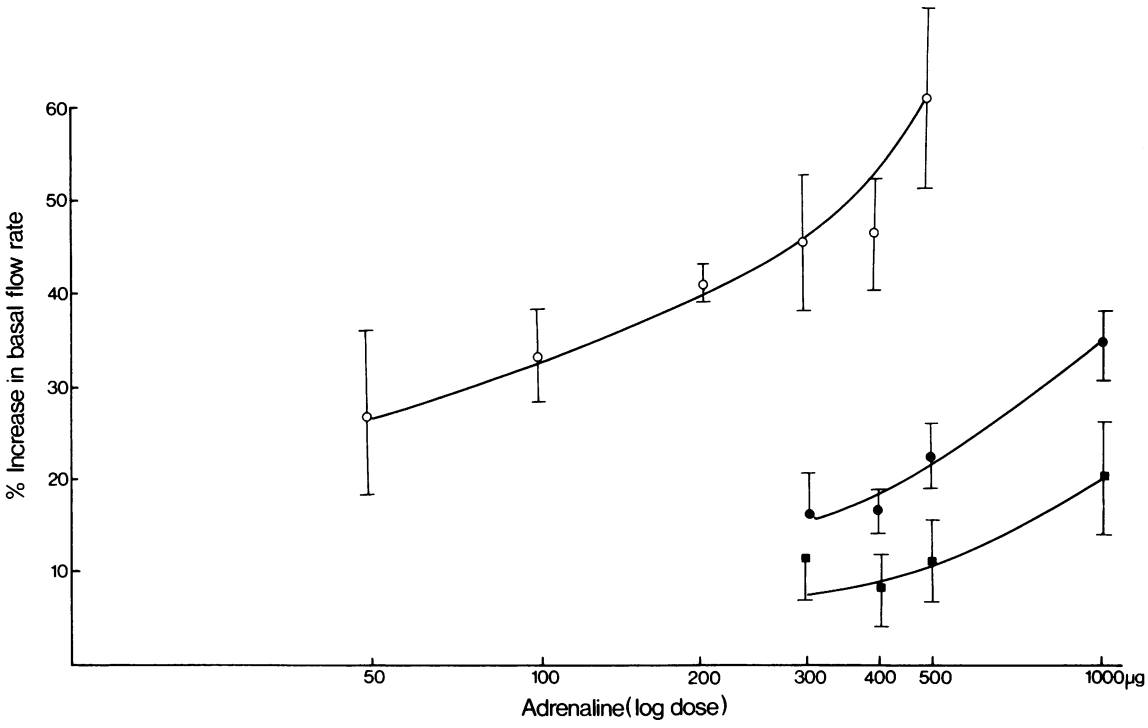

Figure 5 The effect of pretreatment with terbutaline (5 μg/kg) or aminophylline (5 mg/kg) on the response of isolated perfused histamine-constricted guinea-pig lungs to adrenaline. (○) Control; (●) pretreated with terbutaline for 3 days; (■) pretreated with aminophylline for 7 days. Vertical lines show s.e. mean.

prenaline and challenged with adrenaline (Figure 3) or pretreated with salbutamol and challenged with salbutamol.

When the animals were pretreated with aminophylline 5 mg/kg or isoprenaline 5 μg/kg subcutaneously thrice daily for seven days, the log dose-response curve to aminophylline was displaced downwards ($P < 0.01$) (Figure 4). A dose of up to 25 mg did not produce a significant response in the isolated lung preparation.

Similarly, the log dose-response curve to adrenaline was displaced downwards when the animals were pretreated with terbutaline 5 μg/kg thrice daily for three days ($P < 0.001$) or aminophylline 5 mg/kg thrice daily for seven days ($P < 0.001$) (Figure 5).

When the guinea-pigs were left for three weeks after seven days of isoprenaline pretreatment the log dose-response curves of adrenaline in these animals were significantly displaced upwards ($P < 0.01$) compared with the curves of animals left for one week and two weeks after seven days of isoprenaline pretreatment (Figure 6). Recovery from tolerance had begun to develop but was not yet complete.

Discussion

Tolerance Studies

Tolerance to the bronchodilator effect of adrenaline, isoprenaline, salbutamol and aminophylline developed in the isolated perfused histamine-constricted lungs of guinea-pigs which had been pretreated with adrenaline, isoprenaline, salbutamol and aminophylline. The degree of tolerance was dependent upon the amount and the number of doses given during the pretreatment stage. The longer the animals were pretreated and the higher the dose of bronchodilator given (isoprenaline or adrenaline) the greater was the degree of tolerance developed in the isolated lungs. Tolerance could develop to such a degree that regardless of the dose of bronchodilator used to challenge the lungs (adrenaline, isoprenaline or

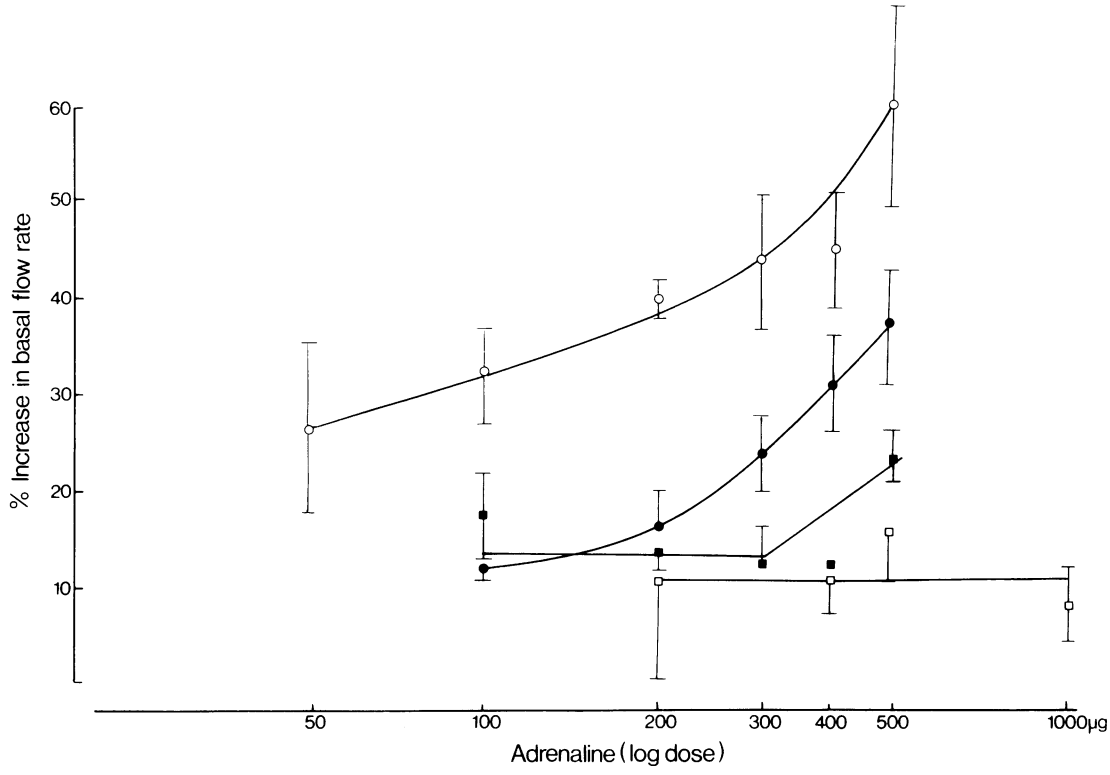

Figure 6 The recovery of the response of isolated perfused histamine-constricted guinea-pig lungs to adrenaline after pretreatment with isoprenaline (5 µg/kg) thrice daily for seven days. (○) Control; (□) animals left for 1 week; (■) animals left for 2 weeks; (●) animals left for 3 weeks. Vertical lines show s.e. mean.

aminophylline) no significant bronchodilation was produced.

Benson & Perlman (1948) and Laurence & Moulton (1960) reported that tolerance developed to the bronchodilator effect of adrenaline after excessive use of this drug in man. Fleish & Titus (1972) showed that tachyphylaxis to the vasodilator effect of isoprenaline developed in the rat aorta and Paterson, Conolly, Davies & Dollery (1968) noticed that tolerance developed to the chronotropic effect of isoprenaline in asthmatic patients taking heavy doses of isoprenaline. Bouhuys *et al.* (1972) also observed the development of tachyphylaxis to isoprenaline in the guinea-pig isolated trachea.

These results suggest that asthmatic patients who are heavy users of bronchodilators may become resistant to these drugs. The value of these drugs in relieving mild asthmatic attacks in such patients becomes less. In an extreme case, a patient may die because the drug he is using has become completely ineffective. A young asthmatic has been found dead clutching an empty bronchodilator aerosol container (Pickvance, 1967). The same conclusion was reached by Herxheimer (1973).

Cross-tolerance studies

Cross-tolerance developed to the bronchodilator effect of adrenaline in the isolated histamine-constricted lung of the guinea-pig when the animals had been pretreated with isoprenaline, terbutaline or aminophylline. Cross-tolerance developed to isoprenaline when the guinea-pig was pretreated with adrenaline and also to aminophylline when the animals were pretreated with isoprenaline. The aminophylline experiments may not indicate whether tolerance is specific and/or non-specific, since aminophylline can be regarded as an indirectly acting sympathomimetic amine because it causes an increase in the release of endogenous catecholamines into the blood circulation (Atuck, Blaydes, Westervelt & Wood, 1967; Westfall & Fleming, 1968; Peach, 1972; Wooten, Thoa, Koplin & Axelrod, 1973). Sympathomi-

metic amines may also act in a way similar to aminophylline, since adrenaline inhibits phosphodiesterase activity (Hitchcock, 1973).

Conolly *et al.* (1971) reported that in guinea-pigs, the mortality from histamine-induced bronchospasm was higher in groups which had been pretreated with sympathomimetic amines than in groups which had been pretreated with saline. They suggested that in asthmatic patients who use sympathomimetic pressurized aerosols excessively cross-resistance develops to both exogenous sympathomimetic amines and to the natural transmitter release by the adrenergic nerves, and that this may lead to a deterioration of the asthmatic state and explain the rise in the asthma mortality rate. The cross-tolerance experiments decribed here may explain the good results which were obtained only after the withdrawal of adrenergic bronchodilator therapy from some asthmatic patients (Keighley, 1966; Reisman, Friedman & Arbesman, 1968).

Recovery experiments

The work described in this paper has demonstrated that the tolerance which developed as a result of the repeated administration of isoprenaline or adrenaline remained for periods of up to three weeks after the last administration of isoprenaline or adrenaline to guinea-pigs.

Mechanism of the development of tolerance

It has been suggested (Robison, Butcher & Sutherland, 1967; Sutherland, Robison & Butcher, 1968; Robison, Butcher & Sutherland, 1971) that the β-adrenoceptor is a component of the enzyme adenyl cyclase and that catecholamines stimulate adenyl cyclase to increase the formation of intracellular cyclic 3′,5′-adenosine monophosphate (cyclic AMP) from adenosine triphosphate (ATP) and that this causes relaxation of the smooth muscle of the bronchi.

Some years ago, Szentivanyi (1968) proposed that asthmatics had defective β-adrenoceptors. Amongst the evidence which supports this hypothesis are the following points: (1) The increase in urinary cyclic AMP in asthmatic patients following adrenaline administration was less than that of normal subjects (Fireman, 1973). (2) The increase in adenyl cyclase activity of leucocytes by isoprenaline from asthmatic patients is less than that of normal subjects (Logsdon, Middleton & Coffey, 1972). (3) Metabolic and circulatory responses to sympathomimetic amines were less in asthmatic patients than in normal subjects (Cookson & Reed, 1963; Lokey, Glennon & Reed, 1967; Middleton, Finke & Arce, 1968; Morris, 1971; Fireman, 1973).

The patients in whom these observations were made were asthmatic and had been using sympathomimetic bronchodilators until a few hours or a few days prior to these studies (Cookson & Reed, 1963; Lokey *et al.*, 1967; Middleton *et al.*, 1968). The resistance of these patients to these drugs mught have been due not to an abnormality of the β-adrenoceptors, but to tolerance which may result from receptor desensitization, produced by the sympathomimetic amines. The idea is supported by the recent work of Nelson (1973) who found that in normal subjects after ephedrine administration, the metabolic and circulatory responses to adrenaline were less than in those who had not received ephedrine. It is also supported by the work of Franklin & Foster (1973) who reported that after pre-incubation of human diploid fibroblasts with isoprenaline for 2-2.5 h, subsequent challenge for 10 min with isoprenaline failed to induce any increase in cyclic AMP. They also suggested that desensitization to isoprenaline in these cells resulted from isoprenaline-induced conformational changes in the receptor which led to their inactivation.

The tolerance to aminophylline which occurred after a guinea-pig had been pretreated with isoprenaline may also support the idea that tolerance to isoprenaline is due to the effect of isoprenaline on adenyl cyclase. It is thought that aminophylline inhibits the phosphodiesterase which breaks down cyclic AMP and that the resulting increased concentration of cyclic AMP causes bronchial relaxation (Robison *et al.*, 1971).

However, if isoprenaline administration had altered the sensitivity of the β-adrenoceptors, and had reduced the activity of adenyl cyclase, this would explain why aminophylline was ineffective, because despite inhibition of phosphodiesterase there would be no increase of cyclic AMP and no muscle relaxation.

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