Pregnancy alters cardiac receptor afferent discharge in rats

TINA HINES AND TRACY M. HODGSON
University of Pittsburgh School of Nursing, Pittsburgh, Pennsylvania 15261

Hines, Tina, and Tracy M. Hodgson. Pregnancy alters cardiac receptor afferent discharge in rats. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R149–R156, 2000.—Reflex effects of cardiac receptor (CR) stimulation are attenuated in pregnant rats. We tested whether CR afferent discharge is reduced during pregnancy by measuring single fiber activity in response to increases in right atrial pressure (RAP) in anesthetized pregnant and virgin rats with sinoaortic denervation. Single fiber activity was isolated from fine filaments of the right cervical vagus nerve. Changes in CR discharge, RAP, and arterial pressure were recorded in response to atrial saline injections (25–300 µl). Resting RAP was similar between groups, and spontaneous CR discharge was similar in pregnant rats (1.95 ± 0.21 Hz) and in low-frequency (LF) receptors in virgin rats (1.30 ± 0.2 Hz). In virgin, but not pregnant rats, a subset (24%) of CR had higher-frequency (HF) spontaneous discharge (9.91 ± 1.19 Hz). During stimulation, the level of RAP above which CR firing increased was significantly higher in pregnant rats, but CR activity was clustered into an LF discharge range. Thus gestation appears to reduce the activity of CR afferents, possibly by increasing stimulus threshold or by selective inactivation of a subset of HF discharging receptors.

PLASMA VOLUME expands by ~40–50% during pregnancy in humans and in rats. In humans, this increase plateaus at midgestation and is maintained until parturition (8). In rats, plasma volume increases progressively until the end of pregnancy (1). Mechanisms that initiate and maintain this marked expansion are not understood, but the maintenance of adequate circulating volume is critical for a successful pregnancy outcome. Contraction of plasma volume is associated with maternal complications, such as preeclampsia (6, 8), and with intrauterine growth retardation in the fetus (8, 18).

Blood volume is regulated by neural and hormonal homeostatic mechanisms. Neural regulation of volume is mediated by a reflex pathway with afferent sensory receptors in the heart, central integration in the medulla oblongata, and efferent projections to the heart and vasculature, particularly the renal vasculature (2, 7). Studies characterizing the afferent limb of this regulatory pathway have indicated the presence of distinct populations of cardiac receptors (CR) that respond to different types of stimuli and have contrast firing thresholds and discharge characteristics. For example, initial studies by Paintal (16) in cats described type A myelinated atrial receptors that fired during atrial contraction and type B receptors that were associated with atrial distention (16). Two subtypes of unmyelinated atrial C-fibers, high (HF)- and low (LF)-frequency receptors, were described by Thore´n (20, 21) and Thorén et al. (23) in rats. Mifflin and Kunze (14, 15), using an in vitro preparation from the rat, also demonstrated two types of atrial receptors, rapidly adapting and slowly adapting. Whether the various subtypes of afferent atrial receptors mediate different efferent reflex effects is unclear, but the evidence points to receptor subtypes that are responsive to distinct types of stimuli and that have characteristic discharge thresholds and firing patterns, supporting the possibility that they could affect reflex effects differentially.

The marked expansion of blood volume early in pregnancy and the maintenance of that increased volume throughout the course of gestation suggest alterations in the reflex neural pathway that regulates volume. Indeed, several investigators have reported that reflex hemodynamic regulation by volume-sensitive stretch receptors in the heart is attenuated in the pregnant rat. Simulation of CR using atrial balloon inflation (11), intravascular volume expansion (17), or atrial saline injections (10) evoked blunted reflex diuresis and natriuresis and attenuated reflex decreases in arterial blood pressure in pregnant compared with virgin rats. In addition, c-fos expression in central medullary nuclei involved in the regulation of blood volume was significantly reduced in pregnant compared with virgin rats in response to atrial balloon inflation (5). In pregnant compared with nonpregnant animals, we have previously characterized discharge in neurons of the nucleus of the solitary tract (NTS) that receive afferent input from CR in response to atrial saline injections (10). Similar changes in discharge frequency in NTS neurons in the two groups were associated with significantly larger changes in right atrial pressure (RAP) and attenuated reflex effects on arterial blood pressure in pregnant rats. These findings suggested that the blunted reflex effects in gravid animals might be due to an alteration in the discharge characteristics of CR or an alteration in receptor subtypes, as suggested by previous investigations, that would contribute to blunted nerve activity in the affer-
ent limb of the reflex arc. To our knowledge, CR afferent nerve activity has not been described in the pregnant animal; therefore, the present study sought to characterize CR afferent discharge in the pregnant rat and to test the hypothesis that CR afferent discharge would increase less in late-pregnant compared with virgin rats in response to increases in atrial pressure.

METHODS

Surgical preparation. Timed (15–16 days)-pregnant (n = 14) and age-matched virgin (n = 14) Sprague-Dawley rats (Zivic Miller, Zelienople, PA) were housed in the Central Animal Facility of the University of Pittsburgh on a 12:12-h light-dark cycle and were fed standard rat chow and tap water for ~4–5 days before studies. On the day of experimentation (gestational day 20), animals were anesthetized with pentobarbital sodium (Nembutal, 50 mg/kg ip) and artificially ventilated via a tracheotomy tube with oxygen-enriched room air. Animals were placed on a heating pad, and temperature was maintained at 37 ± 1°C. Catheters were inserted in a femoral artery and vein for measurement of arterial blood pressure and infusion of drugs, respectively. Supplemental anesthesia was administered as needed (10 mg/kg iv) to maintain a steady arterial pressure (AP) and absence of withdrawal to pinch of the hindpaw. Two catheters (PE-50) were inserted in the right atrium for measurement of atrial pressure and for injection of warmed saline used to stimulate CR. Correct placement of these catheters at the venoatrial junction was verified after experimentation. To eliminate reflex responses mediated by arterial baroreceptors, animals underwent bilateral sinoaortic denervation as described previously (10). Care was taken during this procedure to protect the vagus nerves. Absence of changes in heart rate (HR) in response to increases (4 µg/kg phenylephrine) and decreases (10 µg/kg sodium nitroprusside) in mean arterial blood pressure (MAP) was considered evidence of adequate baroreceptor denervation. The right cervical vagus was isolated, stripped of surrounding connective tissue, and desheathed. The nerve was cut centrally and was positioned on a small mirrored platform in a pool of warm mineral oil fashioned from the surrounding skin. Fibers were split sequentially until a very small bundle of fibers could be isolated and mounted on a bipolar, stainless steel recording electrode. The nerve signal was amplified and filtered between 100 and 3000 Hz (BMA100; CWE, Ardmore, PA), fed to an oscilloscope (model 5111A; Tektronix, Wilsonville, OR) and audio monitor (model AM7; Grass, Quincy, MA), and, along with blood pressure waveforms, was digitized and displayed on an IBM PC (15-KHz sampling rate, Spike2; Cambridge Electronic Design, Cambridge, UK). A window discriminator (BAK Dis-1) was used to isolate single units from recordings containing more than one fiber, and this triggered an event marker on the computer. After experiments, viable pregnancies were verified by visual inspection, weighing, and counting of fetuses. Estrous cycle day was determined in virgin rats by the appearance of a vaginal smear. All experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by the Council of the American Physiological Society and were approved by the Animal Care and Use Committee of the University of Pittsburgh.

Experimental procedures. Plasma volume and hematocrit were determined for each animal at the beginning of experimentation, using the dye dilution technique reported previously (10). After small fiber bundles of the afferent vagus nerve were isolated, the response of those fibers to a rapid bolus injection of 0.9% warmed saline (50 µl) into the right atrium was recorded. Although injection of only 50 µl may have led to an underestimation of the number of responsive CR, it was chosen to minimize the total volume that was injected over the course of an experiment. Nerve bundles were further dissected until a single fiber or a small filament containing three to four fibers was obtained. Activity in one fiber was isolated, and the response of that fiber to another 50-µl atrial injection was recorded. Single fibers that increased discharge with a latency of <400 ms were then stimulated with graded volumes of saline (25–300 µl) at 10-min intervals for as long as a stable recording could be maintained (~2–4 injections). Four-hundred milliseconds were chosen as an approximate cut-off point that allowed data to be acquired for all fibers that could potentially have conduction velocities in the C-fiber range. Baseline AP, RAP, and CR afferent discharge were recorded for 30 s before an atrial injection and for 30 s after the injection to measure the response and to observe the return to baseline levels in all variables.

Data analysis. AP and RAP waveforms were sampled at 200 Hz, and mean values were calculated from the 30-s baseline segment (1-s bins) immediately preceding an atrial injection and from the time segment containing the maximum change in pressure (0.5–1 s) after an injection. HR was derived from the AP pulse, and mean values were calculated as described above. Single fiber CR afferent discharge was analyzed as a rate (10-ms bins), and changes in discharge frequency were calculated as the mean increase in discharge for 1 s after atrial injections compared with the mean discharge frequency in the 30-s baseline recording preceding the injection.

Selection criteria for CR afferent fibers. Two different methods were used to verify isolation of single CR afferent fibers. As described above, during recording, individual fibers were isolated either by microdissection or by using a window discriminator set to capture one spike in a small fiber bundle. After experiments, the raw nerve waveform was reanalyzed using computerized spike sorting (Spike2; CED) to ensure that the rate and pattern of discharge was similar between electronically sorted and discriminated spikes. Fibers that increased discharge by 20% or more were included for analysis in this study. Conduction velocity was estimated for responsive afferents by measuring the latency of nerve responses after the initial increase in RAP and by measuring the approximate distance from the right atrium to the recording electrode. This is a very crude estimation of conduction velocity, since all receptors may not have been situated in the right atrium. Data are reported only for fibers with estimated conduction velocities in the nonmyelinated, C-fiber range (0.4–2.4 M/s). The level of RAP at which a 20% or greater increase in discharge in afferent fibers was noted was assessed by examining the upward slope of the atrial pressure pulse after an atrial injection and recording the pressure level at the time increased discharge was noted. This technique provided an estimate of the pressure level at which threshold was exceeded, but the acute pressure changes evoked by atrial injections were not controlled for rate; thus, it was not possible to determine a true threshold for increased CR firing. In response to atrial saline injections, afferent discharge increased in 46 single fibers in pregnant rats and in 56 fibers in virgin rats. Thirty-two fibers in pregnant rats and 29 fibers in virgin rats (1–4 fibers/rat) were estimated to have conduction velocities in the C-fiber range and are the afferents described in this report. The majority of those fibers that were eliminated from analysis had response times that indicated...
they probably did not originate in the heart. Of the total afferents from which recordings were made, only one in pregnant and two in virgin rats demonstrated pulse-rhythmic spontaneous discharge and were not included for analysis here due to estimation of very rapid conduction velocities. All fibers reported in this study displayed irregular discharge at baseline and in response to atrial injections.

Mean baseline hemodynamic measurements and nerve discharge frequency were compared between groups by unpaired t-tests. Threshold RAP levels and changes in RAP that evoked increased CR firing were compared by ANOVA. Correlations between RAP and hemodynamic variables or CR discharge were determined by least-squares linear regression analysis and were compared among groups by analysis of covariance. Because not all CR afferent fibers were exposed to all volumes, slopes relating RAP to CR discharge were calculated using only fibers that responded to at least two volumes (virgin HF = 5, virgin LF = 15, pregnant = 17). Data are reported as means ± SE, and a P value < 0.05 is considered significant.

RESULTS

Baseline MAP was significantly lower in pregnant (80.4 ± 7.9 mmHg) compared with virgin rats (114.7 ± 2.0 mmHg, P < 0.05). In spite of a significant increase in plasma volume in the pregnant group (pregnant = 16.16 ± 1.75 ml; virgin = 9.03 ± 0.67 ml), mean RAP was similar in the two groups at rest (pregnant = 4.57 ± 1.9 mmHg; virgin = 2.92 ± 3.7 mmHg, P = 0.089). A significantly decreased hematocrit in pregnant rats reflected the increased plasma volume (pregnant = 36.5 ± 0.86%; virgin = 43.5 ± 1.01%, P < 0.05). Pregnant rats were significantly heavier (329.6 ± 9.6 g) and LF CR afferents from pregnant rats were also clustered in the same lower-frequency range and was unrelated to the absolute level of atrial pressure (slope = 0.64 ± 0.25, NS). Afferent discharge in LF fibers in virgin rats was also clustered in the same lower-frequency range and was unrelated to the level of RAP (Fig. 3A; slope = 0.78 ± 0.38, NS). Even though maximum RAP was higher in pregnant than virgin rats, the discharge frequency at which CR firing plateaued in response to atrial injections in pregnant animals was similar to LF receptors in virgin rats (pregnant = 4.32 ± 0.62 Hz; virgin LF = 3.61 ± 0.62 Hz). In contrast, stimulated CR afferent discharge in HF fibers was significantly related to the level of RAP evoked by atrial injections (Fig. 3A; slope = 3.71 ± 1.69, r² = 0.57, P = 0.039). These higher-frequency discharging fibers were the same fibers that had demonstrated HF spontaneous discharge. None of the LF receptors in virgin rats and only one receptor in the pregnant group crossed over from an LF to a HF state in response to atrial injections. Estimated conduction velocities did not differ among groups (virgin LF = 1.28 ± 0.21 m/s; HF = 1.29 ± 0.34 m/s; pregnant = 1.15 ± 0.17 m/s).

The level of RAP at which threshold was exceeded and an increase in CR firing was evoked was significantly higher in pregnant compared with virgin rats (pregnant = 6.23 ± 0.7 mmHg; virgin HF = 2.76 ± 1.89 mmHg, virgin LF = 4.41 ± 0.75 mmHg, P < 0.05 by ANOVA). The RAP at which threshold for increased discharge was exceeded in LF fibers from virgin rats was somewhat higher but not statistically different from that in HF fibers from virgin rats (P = 0.062). As stated previously, the acute changes in RAP associated with atrial injections in this study did not allow an examination of the CR response to a gradual increase in pressure, and we cannot rule out the possibility that differences in the level of RAP at which threshold was exceeded might have been influenced by differences in the rate of injections. Because change in RAP rather than the absolute level of RAP may be a more dynamic stimulus to CR (14), the increase in RAP that evoked an increase in CR afferent discharge was also analyzed, but no differences were found among groups (pregnant = 1.66 ± 0.35 mmHg; virgin LF = 1.64 ± 0.43 mmHg; virgin HF = 2.39 ± 0.95 mmHg, P = 0.33).

Reflex decreases in MAP were significantly larger in virgin compared with pregnant rats in response to
Fig. 1. Data traces of effects of a 100-µl atrial saline injection (arrows) in a virgin rat (A) and a 50-µl injection in a pregnant rat (B). Channels from top to bottom show 1) spikes from a single cardiac receptor (CR) fiber discriminated from the raw trace by computerized spike sorting, 2) raw nerve activity in a vagus nerve filament, 3) right atrial blood pressure, and 4) arterial blood pressure. Both fibers were classified as low-frequency (LF) fibers. Low-amplitude, regular spike in raw nerve trace in A is the electrocardiogram.
increases in RAP (Fig. 4A), and the slope of this relationship was significantly blunted in the pregnant group (pregnant = -0.72 ± 0.36, r² = 0.74; virgin = -4.98 ± 0.98, r² = 0.95, P < 0.05). The correlation between change in HR and change in RAP (Fig. 4B) tended to be blunted in pregnant (slope = -7.6, r² = 0.88) compared with virgin rats (slope = -17.0, r² = 0.65), but the difference was not statistically significant (P = 0.072).

**DISCUSSION**

In this study, the large majority of CR afferent fibers in virgin female and pregnant rats were found to have estimated conduction velocities in the nonmyelinated C-fiber range. This is in accord with the findings of Thorén et al. (23), who identified no myelinated cardiac afferents in male rats. In our study, CR fibers fired with a relatively LF, irregular discharge both at baseline and during stimulation. None of the fibers responded to atrial injections with discharge that was related to the cardiac cycle or with a discharge frequency that was >25 Hz. These observations contrast with the higher maximal firing frequencies and cardiac cycle-related discharge recorded by Thorén et al. (23) in some CR afferent fibers and may reflect methodological differences. Rats in the present investigation were studied with the thorax intact, and RAP was increased using acute injections of saline rather than occluding the aorta to increase left atrial pressure (23). Thus, in the present study, the more rapid pressure increase or the maintenance of negative thoracic pressure could have had an effect on maximal discharge frequency and cardiac-related activity. Lighter body weight could have also contributed to differential findings (7), and there may be as yet unidentified gender differences in CR discharge characteristics.

Despite the relatively low maximal discharge frequency and the irregular pattern of discharge measured in all fibers in this study, there were differences between activity in fibers from pregnant compared with virgin rats. Activity in fibers from virgin rats could be separated into lower- and higher-frequency subsets, both at baseline and in response to stimulation. All fibers with HF discharge at baseline responded to atrial injections with HF discharge, and no LF fibers crossed over to HF activity during stimulation. This supports the concept that the HF receptors represented a distinct subset of CR in virgin rats. In contrast, no HF discharging fibers were observed in pregnant rats, either at baseline or during stimulation. Only LF afferent fibers, similar to the LF fibers in virgin rats, were identified in the late-pregnant rat. Others have reported the existence and characterization of at least two types of CR with nonmyelinated afferents. Thorén et al. (23) described HF and LF receptors in rats based on maximal stimulated firing frequency. LF receptors had higher atrial pressure thresholds than HF receptors and primarily demonstrated irregular rather than cardiac cycle-related discharge during stimulation (23). The level of RAP at which threshold was exceeded in

**Fig. 3.** Relationship between CR discharge frequency and RAP in CR from virgin rats [A; HF (●), n = 5; LF (○), n = 8] and pregnant rats (B; n = 10). Data points represent single fiber responses to graded volume injections (2–4 volumes/fiber). For clarity, not all fibers have been plotted. Slope of the correlation between RAP and discharge frequency was significant (P < 0.05) for HF fibers in virgin rats.
afferent fibers from pregnant rats in the present study was significantly higher than that in HF fibers from virgin rats, suggesting that CR in pregnant rats may be predominantly of the high-threshold, LF type reported by Thorén et al. (23). Indeed, the trend toward a higher RAP threshold in LF receptors from virgin rats and the similarity in peak discharge frequency and firing pattern between these fibers and those from pregnant rats suggests that pregnancy may preserve function in LF receptors while reducing activity in HF fibers.

In response to atrial saline injections, discharge in the HF CR afferents from virgin rats increased in direct correlation with increases in RAP. This is in contrast to the LF fibers from virgin rats and virtually all CR afferents from pregnant rats in which discharge not only increased less in response to atrial injections but increased in a manner that was not directly correlated with the pressure stimulus. Similar stimulus/response characteristics in HF and LF receptors were demonstrated by Thorén et al. (23). Recording from presumed nonmyelinated afferent fibers from the superior vena cava/atrial junction, Mifflin and Kunze (14, 15) also described a class of slowly adapting, HF receptors that increased discharge in proportion to increases in pressure. In preparations from the same animal, they also identified so-called rapidly adapting, LF receptors that increased discharge transiently at a given pressure threshold but that were insensitive to sustained increases in prevailing pressure. Our findings of LF, irregular firing, and a discharge pattern that was unrelated to the prevailing RAP suggest that CR in pregnant rats may be predominantly of the rapidly adapting type. As proposed by Mifflin and Kunze (14, 15), the firing characteristics of LF, rapidly adapting receptors suggest the transmission to the central nervous system of information related to acute changes in rather than absolute levels of atrial pressure. We did not probe the heart to determine the location of the receptors described in this study; therefore, we cannot say that the fibers from which we recorded were located only in the atria, as described by Thorén et al. (23), or in the atroioavlal junction, as described by Mifflin and Kunze (14, 15). Nevertheless, it is of interest that identification of subsets of CR based on firing frequency and pressure threshold is a consistent finding, increasing the likelihood that the reflex differences noted between pregnant and virgin animals may be due to activation of different subsets of receptors. Similar distinct subsets of aortic baroreceptors have also been reported (24).

Mechanisms responsible for the differences in CR activity in pregnant compared with virgin rats await further investigation but may relate to the degree of atrial filling, cardiac compliance changes, or biochemical properties of the receptors, such as changes in ion channels. For example, reduction in extracellular sodium levels, such as occurs during pregnancy, is known to decrease arterial baroreceptor firing (22), and CR discharge might also be altered by this mechanism.

Given the marked increase in plasma volume in late-pregnant rats, it is somewhat surprising that no CR were identified that fired in the HF range at baseline. This finding suggests possible resetting of volume-sensitive receptors during pregnancy. It is known that the threshold for an increase in discharge in CR can reset rapidly in response to an increase in prevailing pressure (15), and there was a trend toward higher RAP in the pregnant group. The similarities between CR in pregnant rats and LF receptors in virgin rats suggest that resetting of LF receptors may occur during pregnancy. LF receptors in both groups increased discharge similarly in response to atrial injections; that is, CR displayed similar LF peak discharge frequencies in response to comparable changes in RAP, and there was no further increase in discharge at higher RAP levels, even in pressures outside the physiologic range in the pregnant group. These findings imply that the afferent signal projected centrally by LF CR is not altered during pregnancy and that differences in reflex effects of CR stimulation in pregnant compared with virgin rats may reflect the absence of HF afferents or other mechanisms as yet undefined in the pregnant rat. Kaufman and Deng (11) reported no difference in unstressed atrial compliance or volume in pregnant compared with virgin rats (11); thus, the high RAP attained during atrial injections in pregnant animals in this study may relate to the injection of a
relatively large atrial volume into an already expanded cardiovascular compartment. An area that remains to be resolved is whether RAP is an adequate index of atrial stretch, particularly in the gravid rat. What is clear is that the high pressures measured in the pregnant rat are not associated with increased CR discharge. Indeed, stimulated CR discharge in the pregnant group plateaued at levels similar to those in LF fibers in virgin animals and within a range of atrial pressures not markedly different from those in virgin rats. Stimulation of CR was associated with attenuation of reflex changes in MAP in pregnant compared with virgin rats. Reflex changes in HR also tended to be blunted in pregnant animals, but the difference was not statistically significant. The lack of significance in the HR response could suggest some gestational alteration in the central processing of the afferent signal, resulting in differential efferent projections to the heart and vasculature. It is also known that direct mechanical activation of the sinoatrial node can reduce HR (7), and this effect may be enhanced in the pregnant rat. Thus changes in HR measured in response to CR stimulation probably involve not only reflex changes in autonomic outflow but also modulation by local mechanical events or possibly by the level of RAP, as suggested by a recent report (3).

In summary, this study has demonstrated a predominance of LF irregular afferent discharge in CR fibers in pregnant and virgin female rats. A subset of HF firing fibers was also observed in virgin rats but not in volume-expanded pregnant rats. Stimulation of CR with atrial saline injections evoked higher levels of RAP in pregnant compared with virgin rats, but the changes in RAP and the discharge frequency at which CR firing plateaued in the pregnant group were similar to LF receptors in virgin rats. CR in virgin rats that had HF activity at baseline responded to stimulation with HF, stimulus-related discharge that accounted for a shift in the pressure-response curve to a more direct correlation between RAP and nerve discharge. These findings suggest that the fundamental changes in autonomic regulation of blood volume during pregnancy may involve the absence of high-frequency, pressure-related afferent discharge in CR and/or a shift in LF receptors toward a higher pressure threshold for stimulation.

Perspectives

Findings from several laboratories indicate that autonomic reflexes are attenuated during pregnancy (4, 10, 13). Some have described gestational alterations in central sites that regulate these reflexes (5, 9). The present observations of altered discharge characteristics in CR suggest that pregnancy may influence nerve activity in the afferent limb of this reflex as well. Mechanisms for changes and the time course of these changes are being defined, but it appears that they may be very early adjustments of pregnancy (11). Autonomic reflexes are attenuated during normal pregnancy in humans, and autonomic dysfunction has been reported in women with preeclampsia (12, 19). Thus elucidation of mechanisms that contribute to the expected blunting of autonomic responses in normal pregnancy is critical in understanding possible pathology in this system that could contribute to conditions such as preeclampsia. The findings of this study indicate that reduced activity in peripheral receptors may be an indicator of an adaptive autonomic response to pregnancy.

We thank Dr. Alan Sved for thoughtful review of the manuscript. This study was supported by National Center for Nursing Research Grant NR-04184 and by the American Nurses Foundation during T. Hines’s designation as an American Nurses Foundation Scholar.

Address for reprint requests and other correspondence: T. Hines, Univ. of Pittsburgh School of Nursing, 440 Victoria Bldg. Pittsburgh, PA 15261 (E-mail: thine@pitt.edu).

Received 5 February 1999; accepted in final form 16 August 1999.

REFERENCES


