This study was design to investigate the "in vitro" effect of bisphenol A (BPA), a component of resin used in dentistry, on viability, and substrate adherence capacity of macrophages. Peritoneal macrophages were obtained from Wistar rats and resuspended in RPMI-1640 medium. Viability was determined by trypan blue exclusion. As a test of macrophage adhesion, the adherence capacity of macrophages to a plastic surface was determined and the adherence index was calculated. Assays were conducted in Eppendorf tubes for 60 min of incubation at 37°C in a humidified atmosphere of 5% CO₂ in air. BPA did not alter significantly macrophage viability at concentrations as high as 10⁻⁵ M, but BPA decreased in a dose-dependent manner the adherence index of rat peritoneal macrophages. Control peritoneal macrophages showed an adherence index = 81.5 ± 7.9%. In the presence of 10⁻⁸ M BPA, the AI of macrophages decreased to 41.4 ± 12.2% (p < 0.05). Higher BPA concentrations (10⁻⁷ to 10⁻⁵ M) also caused a significant inhibition of the adherence index. Half-maximal inhibition (IC₅₀) was obtained at 4.92 ± 0.39 x 10⁻⁶ M BPA. The in vitro study shows that the resin component BPA can alter macrophage adhesion. Taking into account that adhesion is the first step in the phagocytic process of macrophages and in antigen presentation, BPA could inhibit macrophage function and modulate immune and inflammatory responses in dental pulp and periapical tissues.

Today, the monomer 2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy)phenyl]-propane (BisGMA), prepared from bisphenol A (BPA) and glycidyl methacrylate (Fig. 1), is the major ingredient of the resin matrix of most of the resin-based composite restorative materials used in dentistry (1). Furthermore, diglycidyl ether of BPA (BADGE) is also an ingredient of some endodontic sealers used in root canal filling therapy, such as AH26 (2). Polymerization of the monomer BisGMA occurs through the carbon–carbon double bonds of the two methacrylate groups. As polymerization proceeds, diffusion rates of propagating free radicals and unreacted dimethacrylate molecules are drastically reduced, hampering complete conversion of methacrylate double bonds. Thus, as much as 25 to 50% of the methacrylate groups remains unreacted and approximately one-tenth is present as residual monomer. Because of this low degree of polymerization, concerns have been expressed about the leaching of chemicals from material (3). Moreover, cured composites placed in the oral cavity are attacked mechanically and chemically, and the possibility also exists that residual BisGMA may be metabolized to form BPA without prior enzymatic hydrolysis of the ester linkages. Thus, Olea et al. (4) have identified BPA in saliva samples collected during 1 h after placement of a 50 mg fissure sealing in 18 subjects.

The potential impact that this material may have on the biocompatibility of the resin composite and endodontic sealers containing BPA with oral tissues has been of great concern. Pulp studies have shown lack of significant pulpal irritation after the placement of properly sealed resin composite filling (5). However, Jontell et al. (6) have demonstrated that BPA, at low concentrations, increased spleen cell proliferation to concanavalin A.

In previous studies, the existence of a variety of immunocompetent cells in the normal and inflamed dental pulp has been documented, with macrophages predominating (7). Moreover, macrophages are the most dominating immunocompetent cells during all stages of experimentally induced periapical lesions (8). They are known to have several mediator and regulatory functions, and are involved in the entire spectrum of defense reactions.
Besides phagocytizing foreign objects, they produce several biologically active substances (such as enzymes, prostaglandins, and cytokines). Macrophages interact with other cells and components of the extracellular environment by means of adhesion receptors. Adhesion is the first step in the phagocytic process of macrophages (7). Scavenger receptors have been reported to mediate macrophage adhesion to serum-coated plastic surfaces, and adhesion assays exploiting this property have been developed (9).

It is reasonable to assume that resin components such as BPA, following their penetration of dentin or their leakage through apical foramen, may affect the viability or interfere with the function of pulpal and periradicular macrophages. To explore this potential, we have investigated the effect of BPA on macrophage viability by trypan blue exclusion assay and also evaluated macrophage adhesion in vitro to plastic surfaces.

MATERIALS AND METHODS

Chemicals

BPA was obtained from Sigma-Aldrich Co. Ltd. (Gillanham, Dorset, SP8 4JL, UK). 17β-Estradiol, diethylestilbestrol, and RPMI-1640 medium were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

Collection of Rat Peritoneal Macrophages

The protocol was approved by our experimentation committee. Peritoneal macrophages were elicited from Wistar rats. Briefly, each rat was injected intraperitoneally with 5 ml of sterile 6% sodium caseinate. Animals were killed after 4 days by decapitation, and the peritoneal cavity was washed with 10 ml of cold 0.9% NaCl. After a 2-min massage, the cell exudate was removed with a syringe and centrifuged for 10 min at 250 × g at 4°C. The contaminating red blood cells were lysed with cold 0.2% NaCl. The remaining cells were then washed with 0.9% NaCl by centrifugation, resuspended in RPMI-1640 medium, counted, adjusted in the same medium at 2 to 4 × 10⁶ macrophages/ml, and used immediately for experiments. Mean cells per rat varied from 20 to 30 × 10⁶, of which 85 to 95% were macrophages by morphological criteria in Giemsa and Papanicolaou staining techniques. Cell viability was determined by trypan blue exclusion assay. Cell viability found in control samples (90.0 ± 9.5%) and in BPA-containing samples (88.0 ± 6.9 for 10⁻⁶ M BPA to 90.0 ± 8.1% for BPA 10⁻⁸ M) did not differ.

Suspensions of cells containing BPA showed decreased adhesion to Eppendorf tube walls in the model system in a concentration-dependent way. The concentration-effect curve for the effects of BPA on the AI of macrophages is shown in Fig. 3. Control peritoneal macrophages showed an AI = 81.5 ± 7.9%. BPA at 10⁻¹⁰ and 10⁻⁹ M did not significantly alter AI of macrophages (p > 0.05). However, when BPA was added to the incubation medium at a final concentration of 10⁻⁸ M, the AI calculated (41.4 ± 12.2%) was significantly lower than that found in control samples (p < 0.05). Higher concentrations of BPA (10⁻⁷ to 10⁻⁵ M) also caused a significant inhibition of the AI of macrophages. Half-maximal inhibition (IC₅₀) was obtained at 4.92 ± 0.39 × 10⁻⁶ M BPA.

Statistical Analysis

All values were expressed as the mean ± SD of eight separate experiments performed in triplicate. Data were evaluated statistically with ANOVA test. A value of p < 0.05 was considered statistically significant.

RESULTS

BPA did not affect significantly cell viability (p > 0.05) (Fig. 2). By the trypan blue exclusion assay, the cell viability found in control samples (90.0 ± 9.5%) and in BPA-containing samples (88.0 ± 6.9 for 10⁻⁶ M BPA to 90.0 ± 8.1% for BPA 10⁻⁸ M) did not differ.

DISCUSSION

In the present study, it has been demonstrated that BPA decreases in vitro substrate adherence capacity of rat peritoneal macrophages to plastic surfaces.
Macrophages, as members of the mononuclear phagocyte system, use their extensive repertoire of cell surface receptors to interact with their external environment. Exploiting this property, several adhesion assays have been developed (9). We have determined macrophage adhesion to the plastic walls of polypropylene Eppendorf tubes, an adhesion assay previously used to study the effect on macrophage adhesion of different chemicals employed in restorative dentistry and endodontics (10, 11).

Cytotoxicity testing of dental restorative and endodontic materials must be viewed as an assessment of hazards; that is, the potential of the material to cause pulpal or periapical problems. In this context, BisGMA-based composites and endodontic sealers are possible hazards to the pulp and periapical tissues, respectively. The risks that these materials will cause pulpal or periapical toxicity in vivo can be partly estimated by assessing the cytotoxicity of the substances that are released from these materials in vitro and comparing these cytotoxic concentrations with those concentrations that are present in vivo. The resin components of composites have been shown to be cytotoxic in vitro when in sufficient concentrations. The potencies of these substances are quite diverse. However, the cytotoxicity of these substances in usage tests, and therefore the risks of pulpal and periapical toxicity, depends on their ability to diffuse through the dentin and accumulate in the pulp or to leak through the apical foramen to periapical tissues.

Adverse influences of dental materials on biological tissues are generally attributed to either toxic or hypersensitivity reactions. Researchers often fail to ascertain other adverse biological reactions, such as effects on immune functions. With regard to toxicity, tissue damage depends, on the one hand, on the concentration gradient presented to the tissue and, on the other hand, on the cytotoxic potential of the xenobiotic chemical. Because there are no realistic estimates of the concentrations levels at which polymerized resin components may occur in either dental fluid or in the periradicular tissues following the placement of a dental restoration or a root canal treatment, it is not possible to assess if the concentrations tested in our experiments were clinically relevant. However, the IC50 value calculated (4.92 ± 0.39 × 10^-6 M BPA) and the sensitivity of cells to a BPA concentration as low as 10^-8 M suggest that the inhibitory effect of macrophage adherence induced by BPA may have physiological significance in vivo after penetration of BPA into dentin or their leakage through the apical foramen. The ID50 value calculated for BPA in increasing spleen cell proliferation to concanavalin A (50 × 10^-6 M) by Jontell et al. (6) is in good accordance with the IC50 estimated in inhibiting macrophage adhesion.

The inhibitory effect of BPA on macrophage adhesion shown herein, together with the results of Olea et al. (4), who found BPA concentrations ranging from 14.5 to 131.6 × 10^-6 M in saliva obtained 1 h after the application of 50 mg of a sealant containing BisGMA, suggest that the leaching of BPA from composites and sealant applied to tooth cavities could also alter the function of macrophages at the level of oral mucosae.

Analysis of these data suggests that the extractable component from dental composites BPA may not only produce deleterious effects on stromal cells (12), but may also impair the function of pulpal and periodontal macrophages.

Ferracane and Condon (3) reported that 75% of the elutable species are extracted within a few hours and that 95% are extracted within 48 h following polymerization, after which very little will be released. This observation suggests that the risk for toxicity would be immediately after the placement of a composite material and could explain the poor correlation between in vitro cytotoxicity evaluations and in vivo pulp toxicity tests. This prompted the conclusion that resin composites do not seem to provide a chronic source of unreacted BPA to the pulp or other oral tissues. However, in experimental apical periodontitis, the influx of macrophages into the periapical tissues was most evident between 0 and 3 days after the pulp exposure (8). This indicates that the periapical tissues are highly responsive to pulpal injury and begin to work rapidly as a second line of local defense to eliminate noxious stimuli invading the pulp. Thus, if BPA leaks to periapical tissues, it could inhibit macrophage function and delay reparative processes.

On the other hand, cured composite resins placed in root canals are attacked chemically. Enzymatic hydrolysis of metacrylates contribute to the breakdown of composite resins, which are slowly and persistently degraded. Moreover, Kaplan et al. (13) have shown that AH26, an endodontic cement containing BADGE,
disintegrated and lost 1.22% of its mass when stored at 37°C and a relative humidity above 95% for 45 days. This result suggest that BisGMA-based resins used in endodontics could constitute a chronic source of BPA during years.

The mechanism by which BPA decreases macrophage adhesion to plastic surfaces remain unknown. However, there are data that allow us to hypothesize that this inhibitory effect could be mediated by estrogen receptors. First, estrogen receptors have been demonstrated on macrophages (14). Second, estrogens inhibit several functions of macrophages and macrophage-derived cells, such as interleukin-1 production (15), and monocyte- and macrophage-mediated oxidation of low-density lipoproteins (16). Third, the estrogenicity of BPA has been shown (4), and recent observations have increased disquiet about the estrogenicity of bisphenols: a more potent in vivo effect of BPA has been demonstrated, compared with previous in vitro assays (17), and genetic differences in susceptibility to the estrogenic effect of BPA has raised concerns about subpopulations with a higher sensitivity to this estrogen (18). Thus, we can speculate that the effect of BPA could be due to its estrogenicity. Moreover, the inhibitory effect of estrogens, such as estradiol and diethylestilbestrol on macrophage adhesion to plastic surfaces showed in this paper, further supports this hypothesis.

On the other hand, the inhibitory effect of BPA cannot be attributed to a decrease of cell viability, because BPA did not reduce macrophage viability.

Macrophages play an essential role in the immune response of the host to inflammatory and infectious processes, as well as in the reparative process. At the level of pulpal and periapical tissue macrophages, with phagocytosis and antigen presentation, they have a central function in pulp inflammation and in the repair of chronic apical periodontitis (7, 19).

It is tempting to speculate that inflammation affecting pulpal and periapical tissues on many occasions may be the result of a combined effect of material-related cytotoxicity and bacterial infection. There is compelling evidence that many chemicals adversely affect the immune system and that chemically induced immunosuppression is often correlated with decreased host resistance to infectious agents. In this context, other components of dental materials have been found to inhibit macrophage adhesion, such as calcium hydroxide (10), gliberaldehyde, sodium hypochlorite (11), and zinc oxide-eugenol root canal sealer (20). The inhibition of adhesion of pulpal or periradicular macrophages following the use of these materials may enhance the potential for bacterial injury to the pulp and periradicular tissues.

It is concluded that BPA released from resin-based composite materials used in restorative dentistry and endodontics could modify macrophage functions modulating reparative mechanisms and inflammatory reactions at the level of pulp and periradicular tissues.

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References